REMARKS

The Office Action rejected claims 73-78.

I. Rejection under 35 USC 103

The Office Action rejected claims 73-78 under 35 USC 103(a) as being unpatentable over Kamien M. (Aust. Fam. Physician. 1999, Vol. 28, No. 8, p. 817-828, Abstract) in view of Asgharian et al. (US Patent No. 6,139,646), and further in view of Hunt et al. (US Patent No. 6,139,646), and further in view of Rothberg, S. (Science 1967, Vol. 156, p. 90-93). Applicants request that this rejection be withdrawn based upon the analysis provided below.

> Under §103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. *KSR International Co. v. Teleflex Inc.*, 550 U. S. 398 (2007) quoting *Graham v. John Deere Co. of Kansas City*, 383 U. S. 1, 17-18 (1966).

A. Scope and Content of Prior Art

The patent office cites Kamien, Asgharian, Hunt and Rothberg.

Kamien teaches a composition of 15% sodium bicarbonate for use in the removal of human cerumen from the external ear canal.

Asgharian teaches a contact lens cleaning and disinfecting system that, in one example, is formed with a tablet that includes Me-Trypsin, Sodium Bicarbonate and Citric Acid.

Hunt suggests the use of trypsin as one of a great many different enzymes that can be used to aid in the removal of protein deposits from contact lenses.

Rothberg suggests that trypsin can be used to solubilize some types of keratin.

B. Differences Between Prior Art and Claims at Issue

Claim 73 currently reads as follows:

> A composition for assisting in the removal of human cerumen from the external ear canal, comprising about 0.5% to about 15% w/v sodium bicarbonate, about 50 AU/ml to about 500 AU/ml methyl trypsin, about 1% to about 20% w/v glycerin, about 0.001% to about 0.1% w/v benzalkonium chloride, and water, said composition having a pH of about 7.5 to about 9.0;
>
> wherein the composition is configured for, upon location within the ear canal, digesting human cerumen located within the ear canal.

The single and only cited reference that even discusses cerumenolytic solutions is Kamien and it discusses a 15% sodium bicarbonate solution as being the "cheapest and most effective cerumenolytic". Thus, as a starting point, every ingredient other than sodium bicarbonate and water (i.e., methyl trypsin, glycerin, benzalkonium chloride) differentiate the composition of claim 73 from Kamien.

The composition of claim 73 is different from Asgharian in many ways. Asgharian is directed to contact lens cleaning and disinfecting solutions not cerumenolytic solutions. Asgharian uses sodium bicarbonate as an effervescing agent that assists in the dissolution of a

tablet to form a contact lens solution rather than as an aid in digesting human cerumen. Contrary to the assertions of the Office Action, Asgharian never actually suggests that glycerin be used in combination with sodium bicarbonate. The relative amounts of sodium bicarbonate and methyl trypsin used in Asgharian are quite different from those recited in claim 73.

Neither Hunt nor Rothberg remotely discuss cerumenolytic solutions. Hunt stands only for the proposition that trypsin, amongst many other enzymes, can be used to assist in removing protein deposits from contact lenses in a solution containing benzalkonium chloride. There is no suggestion that Hunt discloses any of the other ingredients of the composition of claim 73 or has any relation to cerumenolytic solutions. Moreover, Rothberg merely stands for the proposition that trypsin may be used to solubilize keratin. There is no suggestion that Hunt discloses any of the other ingredients of the composition of claim 73 or has any relation to cerumenolytic solutions.

C. Level of Ordinary Skill in the Art

The level of ordinary skill in the art relative to the invention of the present application would likely be an individual with an advanced degree in the chemical field and/or the biological field with multiple years of experience in designing pharmaceutical compositions.

Obviousness Analysis

Against this background, the proper question to be asked is whether this skilled artisan, faced with the wide range of needs created by developments in the field, would have seen an obvious benefit to upgrading the solution of Kamien by adding about 50 AU/ml to about 500 AU/ml methyl trypsin, about 1% to about 20% w/v glycerin, about 0.001% to about 0.1% w/v benzalkonium chloride, and adjusting the pH to be about 7.5 to about 9.0 in view of the teachings of Asgharian, Rothberg and Hunt.

Applicants suggest that the answer to this question is no for the following reasons: 1) None of the cited references suggest any benefit to the use of methyl trypsin in a cerumenolytic solution much less the use of methyl trypsin in combination with sodium bicarbonate for such a solution; 2) None of the cited references suggest the use of glycerin in combination with sodium bicarbonate; 3) None of the references suggest the use of benzalkonium chloride in a cerumenolytic solution; and 4) The composition of the present invention provides an unexpectedly advantageous cerumenolytic composition.

Methyl Trypsin and Sodium Bicarbonate

The Office Action attempts to suggest that the combination of methyl trypsin and sodium bicarbonate in a cerumenolytic composition is obvious. Specifically, the Office Action reads that:

> a person of ordinary skill in the art at the time the invention was made could have been motivated to modify the cerumenolytic composition as taught by Kamien by adding a preservative composition comprising methyl trypsin according to the teaching of Asgharian et al. and Hunt et al. in order to provide an improved cerumenolytic composition with predictable and additive result of digesting cerumen, because the art clearly teaches the keratinous nature of the cerumen plugs, trypsin's ability to solubilize keratin, and the effective cerumenoltyic actively of 15% sodium bicarbonate solution.

Applicants acknowledge the Supreme Court's statement in KSR that "a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results". *KSR International Co. v. Teleflex Inc.*, 550 U. S. 398 (2007). However, this is not such a combination.

As suggested above, none of the cited references suggest any benefit to the use of methyl trypsin in a cerumenolytic solution much less the use of methyl trypsin in combination with sodium bicarbonate for such a solution. Asgharian is directed to a contact lens cleaning and disinfecting solution. Moreover, Applicants discussed the reasoning as to why the skilled artisan

would not combine the teachings of Asgharian with Kamien in response to the previous office action. That reasoning is incorporated herein by reference.

The Office Action's attempt to supplement the teachings of Asgharian with the teachings of Rothberg and Hunt lacks merit. Neither Hunt nor Rothberg even suggest the use of trypsin in a cerumenolytic solution. Hunt merely mentions trypsin in a laundry list of enzymes that could potentially be used to aid in the removal of protein for contact lenses. (see Hunt col. 7, line 66 to col. col. 8, line 48 all of which discusses various suitable enzymes). Hunt is quite irrelevant for determining ingredients suitable for a cerumenolytic solution.

Admittedly, Rothberg does suggest that trypsin has the ability to solubilize some forms of keratin, of which there are multiple. However, Rothberg does not suggest that trypsin would be useful as a cerumenolytic and this lack of suggestion is important because there are numerous compounds that are known for solubilizing keratin. As evidence of this fact, Applicants submit, in appendix I, A Chapter of Advances in Protein Chemistry, (IX EDITION), 242-99, 1957, which discusses numerous different compounds suitable for solubilizing a variety of different keratins. Without any evidence or reasoning as to why the skilled artisan would specifically select methyl trypsin for a cerumenolytic solution, it cannot be said that such a selection is obvious. Moreover, it would have been even less obvious to combine methyl trypsin with sodium bicarbonate for use in a cerumenolytic solution. Further, it would have been even still less obvious to combine methyl trypsin and sodium bicarbonate in a cerumenolytic solution in the amounts specified in claim 73.

In addition to the above, it is noted that the Office Action suggests that the combination of sodium bicarbonate and methyl trypsin would have produced a predictable and additive result. However, as is discussed in more detail below, the result of this combination is actually quite unexpected and desirable.

Glycerin with Methyl Trypsin and Sodium Bicarbonate

Applicants assert that there is no suggestion in Asgharian to use glycerin in a cerumenolytic solution, much less a suggestion to use glycerin in a cerumenolytic solution that includes both methyl trypsin and sodium bicarbonate. Asgharian, while suggesting the use of glycerin generally, does not particularly suggest its use in combination with methyl trypsin and sodium bicarbonate. Asgharian, relative to the present invention, merely stands for the suggestion that the use of glycerin is a possibility for contact lens cleaning solutions. It does not suggest the use of glycerin in a cerumenolytic solution. As such, it would not have been obvious to the skilled artisan to use glycerin within the cerumenolytic solution recited in claim 73.

Benzalkonium Chloride with Methyl Trypsin and Sodium Bicarbonate

Applicants assert that there is no suggestion in Asgharian or Hunt to use benzalkonium chloride in a cerumenolytic solution, much less a suggestion to use benzalkonium chloride in a cerumenolytic solution that includes both methyl trypsin and sodium bicarbonate. Asgharian and Hunt, at best, suggest the use of benzalkonium halide or benzalkonium chloride as a possibility for contact lens cleaning solutions. They do not suggest its use in combination with methyl trypsin and sodium bicarbonate. Moreover, Asgharian does not suggest the use of benzalkonium chloride in a cerumenolytic solution. As such, it would not have been obvious to the skilled artisan to use glycerin within the cerumenolytic solution recited in claim 73.

Summary

As stated in KSR, "A patent composed of several elements is not proved obvious merely by demonstrating that each element was, independently, known in the prior art." 550 U. S. 398 (2007).

For the present application, the Patent Office has shown no more than the existence of the ingredients of the composition recited in claim 73. The Patent Office has provided no evidence

of the use of at least three of the ingredients (methyl trypsin, benzalkonium chloride and glycerin) of the composition of claim 73 in a cerumenolytic composition. Moreover, the Patent Office has failed to show the relative amounts of the ingredients recited in claim 73 are within the prior art. In view of the above, Applicants request that the rejections of the claims of the present application be withdrawn.

Secondary Consideration - Unexpected Advantage

In addition to the failure of the Patent Office to show that the cerumenolytic composition of claim 73 is obvious, Applicants believe that such composition is particularly non-obvious because of the unexpected advantage that it provides. On this matter, the application reads:

> In addition, it was unexpectedly discovered that composition D exhibits a synergistic effect in removing both human and artificial cerumen. Referring again to Table 8, the absorbency measurements for the bicarbonate component D_1 of Composition D, the proteolytic enzyme component D_2 of Composition D, and the vehicle V_2 of Composition D are also provided. As shown by Table 8, the sum of the absorbency measurements of the bicarbonate component D_1 and the proteolytic enzyme component D_2, subtracting any contribution from vehicle V_2, is significantly less than the absorbency measurement for Composition D, subtracting any contribution from vehicle V_2, for the protein component and the lipid component of both human and artificial cerumen.

This unexpected advantage of the invention of claim 73 provides substantial evidence of the patentability of that claim. With the cited prior art suggesting only the use of sodium bicarbonate for cerumenolytic solutions, the discovery of synergy provided by the combination of ingredients of claim 73 is an advance and discovery that is truly valuable and represents a significant step in the preparation of cerumenolytic solutions. Such a significant step is believe to be worthy of patent protection. As such, allowance of the claims of the present application is respectfully requested.

Applicants also note that claim 78, which is dependent upon claim 73, specifically recites the synergy discussed within the application. The Office Action fails to provide any basis for the rejection of claim 78. As such, Applicants suggest that the Office Action has failed to establish a prima facie case of obviousness against claim 78. On this basis, Applicants request that the rejection of claim 78 be withdrawn. Further, Applicants offer to add the language of claim 78 to claim 73 if claim 78 were deemed patentable by the USPTO.

Respectfully submitted,

Alcon Research, Ltd.

March 19, 2009

Scott A. Chapple
Reg. No. 46,287

Address for Correspondence:
Alcon Research, Ltd.
IP Legal TB4-8
6201 South Freeway
Fort Worth, TX 76134-2099
Tel: 817-615-5288
Fax: 817-551-4610

Docket #2399 F US

The Formation, Composition, and Properties of the Keratins

By WILFRED H. WARD and HAROLD P. LUNDGREN

Western Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Albany, California

Contents

I. Introduction

Although the keratins occur widely, usually as protective coverings in the vertebrates, there exists a remarkable lack of information regarding their formation, detailed structure, and composition. A reason for this imperfect knowledge lies in the fact that the keratins are generally difficultly soluble and morphologically nonhomogeneous and consequently are not as amenable as most proteins for study for purposes of characterization. Interest in the keratins is increasing along with the refinement of techniques for study of solid materials, and for isolation, controlled degradation, and analyses of proteins and protein fragments.

This review is limited in scope; it is intended to bring together current information on keratin formation and composition and to point out relationships of this information to the present conception of keratin structure.

II. Keratinization and Morphological Structure

In considering keratinization we shall discuss how the form of typical keratin structures arises in a living animal and also how the substance of the keratins, with characteristic properties of insolubility, elasticity, oriented structure, and sulfur content, arises on a microscopic scale from precursors that do not have these properties.

1. *Physical Aspects of Keratin Formation*

The vertebrate epidermis and other keratin structures, which are derived from it, grow by cell division confined to the innermost part of its unkeratinized (Malpighian) layers (39). Two significant features are the progressive outward displacement of the cells and the formation of fibrous keratin from or around a fibrous structural precursor, the tonofibril, extending through and between cells (32). In keratinization the growing cells are displaced from their original source of biochemical raw material. Their metabolism is correspondingly altered. As the cells enter the cornified layer the nonprotein constituents are reduced in proportion and, for the most part, disappear entirely. Fibrous elements arising in the growing layer become consolidated (to some extent oriented), concentrated, insolubilized,

hardened, and dried. In hair they show the characteristic α X-ray pattern and optical birefringence even before keratinization. The fibrous elements are formed in rough alignment with the direction of cell movement. The alignment is later modified by changes in the size and shape of the cells. The original orientation is retained and improved when the cells become elongated in the direction of growth, as in the cortex of hair and wool. When the cells become flattened, it is modified so that fibers lie in all directions at approximately right angles to the direction of compression, as in the skin.

2. *Biochemical Environment for Keratin Formation*

As a general rule it can be stated that the cell layer undergoing active division contains a great variety of biochemical substances known to take part in normal metabolic processes. These are diminished or absent in the cornified layers; and the decrease is particularly complete in the case of hard keratin. Metabolites that have been observed to be present in the layer of growth but absent from the keratin itself include deoxyribonucleic acid, ribonucleic acid, ascorbic acid, alkaline phosphatase, and glutathione (24). Wool root extracts (18) have been found to show the presence of free glutamic acid, glycine, alanine, the leucines, and serine. Basic amino acids were regularly present. In one case lysine and arginine were identified separately. Glutamine, aspartic acid, and cysteic acid occurred in fewer instances. Components chromatographically similar to taurine, ornithine, α-aminobutyric acid were also observed. Most of the acids found are major components of wool keratin. It is suggested that the irregular occurrence of some of these is due to the balance of a more or less steady demand and a fluctuating supply. The absence of free proline and cystine or cysteine is notable. Although one third of the sulfur content of the root is in the reduced state, only 4% was water soluble and dialyzable.

The acids of the Krebs cycle have also been demonstrated in wool roots, but not all simultaneously. Citric, α-ketoglutaric, succinic, and malic acids were found in one extract and succinic and fumaric acids in another. A balance between a variable supply and a more uniform demand is again suggested.

The copper content of the wool roots is about 40 p.p.m. of dry tissue, averaging about six times higher than in the keratinized fiber.

The various enzymes or enzyme systems that have been demonstrated in wool roots include alkaline and acid phosphatases, pyrophosphatase or adenosine diphosphatase or both, a system converting metaphosphate to orthophosphate, thymonucleic acid depolymerase, phosphorylase, a dehydrogenase (with an unidentified natural substrate) acting on some L-amino acids and glycine, catalase, and a system catalyzing the absorption

of carbon dioxide. Attempts to demonstrate the presence of lipase, cystine decarboxylase, cysteine oxidase, phosphatases acting on lecithin or phytin, and tyrosine oxidase were negative. The alkaline phosphatase was shown to be concentrated mainly in the root sheath with possibly a higher concentration near the zone of keratinization. On the other hand, the acid phosphatase occurred mainly between the cortical cells and only in the portion of the fiber actually undergoing keratinization. These observations are exceedingly interesting even though they are preliminary and do not give an integrated picture of keratin synthesis. The presence of the acids of the Krebs cycle and of the most nearly related amino acids glutamic acid and alanine, and the fixation of carbon dioxide are in agreement with the idea that these substances may participate in protein synthesis. The presence of the various enzymes transferring phosphate bonds may be related to the use of phosphate bond energy for peptide bond synthesis. However, the specific occurrence of acid phosphatase solely in the region of keratinization suggests a possibly important special role for this enzyme. Since no specific catalyst for disulfide bond formation was demonstrated, it is possible that the copper present may assist as an inorganic catalyst. It is notable that only a small part of the total copper is transferred into the final keratin.

In addition to the disappearance of various nonprotein substances in the course of keratinization, two definite chemical changes have been demonstrated in the fibrous elements (2). After these elements are first laid down they develop a special affinity for acid dyes. As keratinization proceeds optimally, they assume instead preferential affinity for basic dyes and finally for "nitro" dyes such as picric acid. The second change is the incorporation of sulfur, first in the reduced form, which is then oxidized as one of the principal mechanisms of insolubilization.

3. *Soft and Hard Keratins*

Two types of keratin can be distinguished on the basis of physical character, histology, and chemical composition: soft and hard. The outer layer of the epidermis, itself, is the type example of soft keratin. Such material is most conveniently isolated from the thickened skin of cattle noses and lips or horse burrs. Horn, nail, claw, and hoof are typical examples of hard keratin. Hair, wool, and feather are classed as hard keratin but are associated with soft keratin in the medulla, where present, and in the central canal. In fact, the hard keratins are typically associated with soft keratin structures such as the eponychium (the cuticle at the base of a fingernail is a familiar example) and the inner root sheath.

a. Features Characteristic of Soft Keratin. The soft keratins have a relatively low degree of consolidation. As a result they continuously, spon-

taneously slough the outermost layers. Chemically, skin keratin is distinguished by a sulfur content of the order of 1% of the dry weight and a lipid content of about 4%. Glycogen is reported present. The sulfur is rather evenly distributed between cystine and methionine (both combined to form protein). The sulfur and tonofibrils are concentrated near the cell boundaries. The fatty material includes fatty acids, phospholipid, and sterol. These properties are considered typical. Block (6) has suggested that skin keratin is characterized by more nearly equimolar proportions of the amino acids lysine and arginine than hard keratins, which are higher in arginine. Using this criterion he groups mammalian skin keratin with whalebone (baleen), tortoise shell, and pelican excrescence as pseudokeratins. Histologically, soft keratin shows a characteristic, abrupt transition from the growing germinal layer to the keratinized layer through a granular layer, containing irregular particles staining with basic dyes, and a narrow, glassy layer without visible detail.

b. Features Characteristic of Hard Keratin. Some differences between hard and soft keratins have already been suggested. Hard keratin is characteristically higher in sulfur, having up to 5% (9) (43), preponderately in the form of combined cystine. The sulfur content is quite variable even for a single kind of keratin material such as sheep's wool, for which the normal range is from 3% to 4% (3). Nonprotein constituents are very low. Appreciable amounts of lipid and glycogen, for example, are not present. Arginine is present in larger amount and in larger proportion to lysine than in soft keratin. The hard keratins form more coherent structures with higher tensile strength. Under the microscope the transition from the growing layer to the fully hardened product is seen to take place more gradually over a much wider region in which the sulfur content is increased markedly. Finally, the structures forming hard keratin, although continuous with the skin, are more highly complex and specialized. They regularly show modified features such as a thinner underlying basement membrane and a more liberal blood supply at the site of growth, facilitating more rapid metabolism. The evidence suggests that hard keratin arises by adaptation of the soft keratin mechanism to expedite the incorporation of sulfur and the formation of keratin in quantity.

It is desirable to clarify further the use of *soft* and *hard* to describe keratins. The incompletely keratinized parts of tissues producing either soft or hard keratins as defined above yield substantial fractions of protein soluble in solvents such as concentrated aqueous urea. These parts might also be called soft or unhardened. To avoid confusion it is better to refer to them as *prekeratin*, indicating their relationship as precursors to both soft and hard keratins. Rudall's epidermin (64) is an example of prekeratin. In addition, fully developed keratins frequently show portions

differing in degree of consolidation, with associated differences in composition, swelling behavior, dye affinity, and other properties. The transition between such portions is sometimes gradual and indefinite, sometimes quite abrupt. Segments of wool fibers differing in degree of consolidation in this way have also been described as soft and hard. This distinction has a different basis from the classification developed in detail above, although it may overlap in some cases. Mercer's (50) designations *ortho* (less consolidated) and *para* (more consolidated) for these segments are convenient. These other variations in and among keratins will be discussed further at appropriate places.

4. *Keratinization of Soft Keratins*

In the process of keratinization of soft keratin the most characteristic histochemical feature is the abrupt disappearance of the sulfhydryl reaction at the boundary between the granular and glassy layers. This and additional evidence (64) indicates that the proteins of the growing layer are keratinized at least in part by the oxidation of sulfhydryl to disulfide. Presumably, the protein chain structure is stabilized by covalent cross-linkages. This hypothesis is supported by a large number of observations of increased enzyme digestibility and solubility of keratins when the disulfide bonds are broken by reduction or oxidation. The process is common to both types of keratin, but is less effective in soft keratin because of its lower content of cystine. It is of special interest that the sulfur content of the fibrous protein of skin, its α, X-ray–determined configuration, and tensile properties, including its ability to undergo transformation to the β form, are practically the same in the growing and keratinized layers. Nonfibrous protein, about 9% of the total protein, increased in sulfur content in the course of keratinization to 2.9%, approaching the sulfur content of hard keratin.

5. *Keratinization of Hard Keratins*

A particularly characteristic difference between hard and soft keratins is evident in the histochemistry of keratinization. The hard keratins show a much broader transition region between the germinal layer and the cornified structure. Although the germinal layer shows the presence of some free sulfhydryl groups, their number is greatly increased in the transition region, together with the total sulfur content. The change in total sulfur is much greater than that observed with skin. In fact, the available data show that the sulfur content of the growing layer is about the same for both soft and hard keratins. At least part of the fibrous structure is laid down before the incorporation of the bulk of the sulfur, which may either (a) be substituted into the existing fibrous protein, (b) form part of a

cementing link joining existing protein chains, which can then also cross-link, or (c) form part of separately formed protein which is then laid down around the existing fibrous scaffold.

6. *Development of the Wool Fiber*

Wool fiber structure is of special interest because of its technical importance. For this reason its development is known in greater detail than that of most other keratins and can be used to illustrate some of the generalizations of the preceding summary.

Wools have recently been distinguished according to the nature of the follicle from which they are derived. Two types of follicles have been recognized—the primary and secondary. The *primary follicles* are first to form. They are arranged in groups of three of which the central member produces fibers having sickle-shaped tips. These fibers appear before the fibers from the lateral primary follicles, which produce fibers having many curls and which have been termed "early curly tips." The *secondary follicles* similarly produce fibers of two types; the early secondary follicles produce fibers which have fewer curls at the tip and which are termed "late curly tips." The late secondary follicles produce fibers which are either straight or with few and very small curls. These are called histerotrichs. The process of keratinization is probably very similar in the primary and secondary follicles.

a. The Follicle. A wool follicle is diagrammed, roughly to scale, in Fig. 1. This tube, in which the fiber grows, forms as a finger-like projection of the epidermis slightly inclined from the perpendicular and growing down about 2 mm. into the dermal layer. The tip partly surrounds a papilla of fibrous connective tissue that is particularly well supplied with blood vessels and has a larger concentration of cell nuclei than the dermal tissue underlying the skin epithelium. The collagenous basement membrane separating the dermal from epidermal tissues is especially thin or absent from around the papilla. These features adapt the base of the follicle around the papilla to be a site of rapid localized cell division and growth. In mature sheep the papilla is eccentric, so that the bulb surrounding it is often bent at a sharp angle to the axis of the emerging fiber.

The cells in the bulb in the region of primary growth near the papilla are alike, with indistinct boundaries and a crowded appearance. In the wide part of the bulb they differentiate into several distinct layers which become the various layers of the fiber and its root sheaths.

b. The Root Sheaths, Their Relation to the Fiber Structure. The root sheaths consist of four distinguishable cell layers. Three of these layers constitute the inner root sheath, which undergoes the characteristic abrupt transition into soft keratin somewhat above the bulb. The fourth layer,

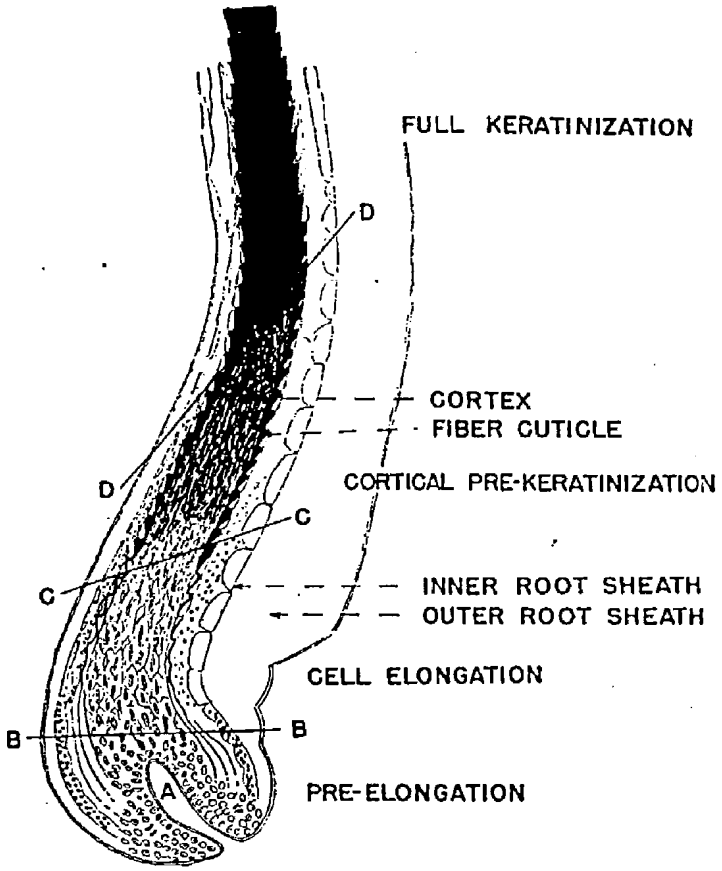


FIG. 1. Diagram of wool follicle (after Auber). The figure represents a somewhat foreshortened longitudinal section of the innermost one-half to one-third (about 0.6 to 1 mm.) of a nonmedullated wool follicle. Changes in cell structure and histochemistry in the course of keratinization are indicated. Four stages are roughly distinguished. These are, for the cortex:

Pre-elongation Region, in the immediate neighborhood of the papilla (*A*) in the bulb. Cell nuclei, some dividing, are prominent. There is a small concentration of free thiol (—SH).

B-C Region of Cellular Elongation above the bulb. Cell boundaries of elongated, polyhedral cells become distinct. Many thin parallel fibrils appear, oriented roughly longitudinally, and coalesce. They stain with acid dyes and are optically birefringent. At this level the fiber shows its characteristic α X-ray diffraction pattern. The concentration of free thiol increases remarkably.

C-D Region of Cortical Prekeratinization. The fibrils and, slightly later, the whole cell assume an affinity for basic dye. Nuclei are reduced to thin rods. The fibrils form an indistinct and irregular mosaic, obscuring the cell boundaries. The fiber cuticle and epicuticle are already completely hardened at about this level (*C*).

Fully Keratinized Region (of the fiber), shown in solid black. The usual asymmetry of keratinization is indicated. At the limit of full keratinization the concentration of free thiol disappears. The fiber no longer shows nuclear remnants, fibrillar structures, or cell boundaries except by special treatment. The keratin stains with nitro dyes and shows characteristic relative resistance to enzymes, long-range reversible extensibility, and supercontraction with disorientation of structure in water at characteristically high temperatures. The diameter is reduced about 25% from that at the beginning of "Prekeratinization" (*C*).

The uppermost line is drawn approximately parallel with the skin surface.

the outer root sheath, is continuous with the growing layer of the skin epidermis. It forms typical keratinized skin lining the upper end of the follicle. The root sheaths are concerned with the structure of the wool fiber in three ways.

The outer root sheath is greatly thickened in the region above the bulb. When, as in most cases, the follicle is bent just above the bulb level, the thickening is more pronounced on the side to which the bulb is deflected. Keratinization of the fiber takes place sooner on the thinner side. It is not known, however, whether this circumstance actually indicates a causal relationship.

A second possible way in which the wool fiber structure may be affected by events concerning the root sheath is suggested by the way the inner root sheath disintegrates at its upper limit, about one-third of the way from the skin surface to the papilla. Near this level the cells of the inner layer, the root sheath cuticle, separate. The other layers lose their base-binding character, merge, thin out, and disappear. This reabsorption of the inner sheath is inferred to be due to a chemical agent, possibly a keratolytic enzyme derived from the outer root sheath. In pathological conditions the cortex may even be attacked at this site. This raises the question: "To what extent can the wool fiber be affected under usual conditions? How is it normally protected?" The possibilities for normal protection seem to be either that the agent is specific for the keratin of the inner root sheath, or that its quantity is just sufficient to dispose of this sheath, or that the fiber is ordinarily protected by the epicuticle.

Finally, the inner root sheath cuticle is in close contact with the cuticular layer of the fiber. The surface configuration of the fiber cuticle, which is important for the felting and spinning properties of the fiber, is determined by the relative turgidity, plasticity, and progress of keratinization in the two cuticular layers and by shear stress and pressure transmitted from the outer layers.

c. The Fiber Cuticle. The development of the fiber cuticle proceeds in the following manner. The two cuticle layers are first distinguishable from neighboring cells at about the level of the widest part of the bulb. Here, in the sheep, they consist of single layers of large, flat, and oblong cells with the longer axes vertical. As the cells are forced into and beyond the constricted region above the bulb, the root sheath cuticle cells bulge downward and toward the axis. This change is accommodated by lesser relative turgidity of the fiber cuticle, which is more advanced in the series of changes leading to keratinization. The downward bulge of the root sheath cuticle cells, which act as a mold for the fiber cuticle, is attributed to the faster relative movement of the outer layers of the inner root sheath as it leaves the bulb region. This relative motion is made more effective

by the early hardening of the outermost layer of this sheath. The upper ends of the fiber cuticle cells of fine wool project sharply outward, overlapping the cells above only slightly and giving a serrated edge. The cuticle of coarse fibers shows a more scalloped profile. Keratinization occurs while the fiber cuticle is in this form. The projecting tip and then the rest of the cell develop an affinity, successively, for basic, then for nitro dyes. The root sheath cuticle hardens at a higher level.

d. The Epicuticle. Part of the chemical stability of wool, animal hairs, feathers, and human skin, and possibly other keratins results from a chemically resistant outer covering, the epicuticle. In the case of wool, this membranous structure is generally 50 to 250 A. thick and amounts to 0.1 to 0.2% of the whole. It is isolated typically either after treating wool with chlorine water or bromine water, which loosens it from underlying layers, or by dissolving the bulk of the wool with dilute sodium sulfide. Under certain conditions chlorine and bromine have been found to differ in the thickness of the film released. It is probable that under certain conditions the isolated membranous material may include underlying cuticular layers. Treatment with proteolytic enzymes after preliminary treatment with phenol has also been used. Since epicuticle preparations are difficult to free unequivocally from other wool constituents and from foreign matter, it is correspondingly difficult to determine the chemical composition. The resistance to digestion by proteolytic enzymes made it seem likely that the epicuticle was nonprotein. Further study now has established that the material is protein, and amino acid composition has been partly determined. This noncellular, protein membrane has been shown to exist at all levels above the approximate zone of keratinization of the rest of the fiber (67) (68). It is therefore not secreted by the sebaceous gland. This fact is confirmed by the absence of sebaceous and "sweat" glands from the numerous secondary follicles producing normal wool.

e. The Cortex. The cortex forms most of the fiber. It is in direct contact with the cuticle. The cells from which it arises are like the others in the lower part of the bulb except that their nuclei appear elongated, as if especially crowded, in the layer next to the papilla. As they move upward they enlarge, elongate greatly, and are compressed laterally.

The cell boundaries become distinct slightly below the constriction at the top of the bulb. At this level a system of very numerous, uniformly distributed, fine fibrils staining with acid dyes appears, roughly oriented in the direction of fiber growth.

The fibrils then gather into small, uniformly distributed groups that coalesce into sharply outlined, refringent rods, bent slightly around the nucleus and meeting the cell boundary at an oblique angle. The boundaries are linear and well marked by fibrillar structures.

At this level, slightly above the top of the bulb, the fiber structure has already attained its final form insofar as can be shown by optical birefringence and an oriented α X-ray pattern (48). This region is not yet keratinized, for it is readily dissolved by dilute alkali, trypsin, or concentrated aqueous urea. By 30 seconds heating in water at 90° C., the X-ray pattern is disoriented and changed to the β form; the birefringence is destroyed without change in shape. A high concentration of free sulfhydryl groups can be demonstrated (24), beginning near the level of fibril formation and tapering off in the region in which keratinization is completed. Fiber formation may therefore be considered a process distinct from keratinization.

Hardening of the fibrous structure is progressive, but two histochemical steps may be distinguished. In the first, the fibrillar structure changes its dye affinity so that it binds basic dyes. The change occurs first near cell boundaries. At a higher level the structure forms an indistinct, irregular network partly obscuring the cell boundaries. The substance between the fibers also comes to bind basic dyes. The nuclei are transformed into homogeneous appearing, irregular, thin rods.

The final step in keratinization of the cortex occurs about one-third of the way from the tip of the papilla to the skin surface. At this point the fiber, although still acid in character, develops an affinity for picric acid and other nitro dyes. The sulfhydryl reaction disappears. In these two steps, the fiber attains its final chemical and thermal stability. It then can be disoriented, in water, only with supercontraction and at temperatures above 100° C. The various steps in hardening tend to occur first at the edge of the fiber, progressing toward the center. If the fiber is, as usual, eccentric, keratinization is also biased so that it tends to occur first near the thinner side of the follicle wall.

f. The Medulla. Thick fibers, especially, may also have a central medulla: a filamentous tissue, alkali-resistant but low in sulfur and having a high proportion of air space. The medulla width is variable, even in a single fiber, so that a small medulla is often discontinuous. Medullation is of practical interest because it decreases the commercial value of the wool. It is also of special interest in the study of keratin structure because the medulla can be formed from cells initially of the same type and arising at the same site as those becoming the cortex. The process is in part under nutritional control. Since the medulla and cortex differ strikingly in physical character and composition, medullation affords a criterion for evaluating biochemical and biophysical requirements for the formation of hard keratin.

The medulla arises from the central part of the papilla. Its width is in part related to the papilla size and shape. The boundary between cortex

and medulla is not fixed. The cells forming the medulla ordinarily do not divide after leaving the basal layer; however, cells from the adjoining layer that becomes the cortex do divide normally up to about the level of the wide part of the bulb. Some of these cells move over into the region of the medulla and develop as medulla thereafter.

Cells developing into medulla are first distinguished, in the bulb region, by their larger size and more horizontal orientation than cortical cells at the same level. The medullary cells enlarge greatly by imbibition. Their turgor minimizes deformation as they are forced out of the bulb. It may be inferred that, especially in comparison with the cortical cells, their osmotic pressure resulting from soluble but nondiffiusible components is high. Water is therefore transferred from the cortex to the medulla in this region. A plausible explanation for such a difference in osmotic pressure is that the cortical cells are metabolically more active and therefore more advanced in condensing their available substance into insoluble and high molecular weight material. As an additional consequence, the medulla can be expected to lose any diffusible metabolites that are being used up at a faster rate in the cortex.

The cells of the medulla have, at any rate, a very low solid content. In the course of keratinization they undergo the formation of vacuoles eventually occupying most of their volume. The remnants of the cell body among the vacuoles form filamentous bridges initially joining the cell nuclei with one another and adjoining cortical cells. In hardening, these filaments may coalesce to varying degrees. They develop an affinity for acid dyes, as in the first stage of keratinization of the fibers of the cortex and cuticle. There are no further changes in dyeing properties. The relatively small sulfhydryl content disappears without showing the massive increase characterizing the cortex. The fibers show the β X-ray diffraction pattern rather than the α form characteristic of the cortex.

It may be concluded that medulla formation occurs when certain essential nutrients, especially sulfur-containing substances, become relatively unavailable to the tip of the papilla. This condition may result because of high metabolic activity of the epidermal cells in contact with the lower parts of the papilla, such that the supply of metabolites to the tip is depleted, or because of localized distribution of the blood supply in the papilla. These effects are aggravated if the papilla is large or if the supply of nutrients is deficient. Therefore in medullation two nutritional effects are in balance. At least in some species and under certain circumstances, optimal nutrition increases follicle size, increasing medullation. On the other hand, specific nutritional deficiencies can produce medullation (46). The medulla may represent a modification of the fundamental fibrous scaffolding characteristic of epidermal tissue under conditions such that it

is not directly supported by supplementary keratinization, but is largely prevented from consolidating by its attachment to the surrounding tube of hard keratin.

7. *The Relation between Wool Fiber Shape and Internal Structure*

Fiber crimp is a property of practical value not only because of its relation to spinning properties but also as an indication of physical quality. Horio and Kondo (33) have presented a clear description of the relation of crimp to a bilateral division of the cortex into segments differing in chemical and physical properties. Their observations have been confirmed or supplemented in other laboratories. An interesting example is shown in Fig. 2. Mercer (50) has correlated a great deal of evidence concerning internal structural details of wool. He proposes to describe the less hardened cortical segment as orthocortex. This is more susceptible to swelling and solubilization and dyes relatively more readily with basic dyes and less readily with acid dyes. The more consolidated segment is denoted paracortex.

The formation of crimp or a tendency to crimp is readily related to asymmetry in the follicle. Of various unsymmetrical features, the most significant are probably the bend at the top of the bulb and the lateral position of the papilla. The following hypothesis of their origin and effects has been assembled by Auber (2). The primary process resulting in crimp is a localized region of maximum rate of cell division to one side of the papilla axis. The preponderate growth on this side directly produces the deflection of the bulb and the eccentricity of the papilla. As a result of the bulb deflection, the fiber presses against the follicle wall on the side of more rapid growth so that the outer root sheath is much thinner on this side. The fiber keratinization is more advanced near the thinner side of the follicle wall; at this side the fiber is more accessible to an inductive effect of the dermal tissue. The chemical readjustments in the course of keratinization progressively contract the fibrillary structures by establishing and increasing the amount of α-folding. Because the progress of keratinization is more advanced on the thin side, the contraction is one-sided and bends the fiber and the follicle so that they are concave toward the thin side. The progress of keratinization across the fiber locks in stresses due to the contraction. The mechanical stress on the follicle dislocates the region of maximum cell division, producing corresponding changes in bulb deflection, fiber eccentricity, and the segmental keratinization. Successive portions of the fiber accordingly become bent in different directions. Periodically varying or uniform conditions of growth result in regular crimp. Small crimp period is related to a relatively short region of keratinization.

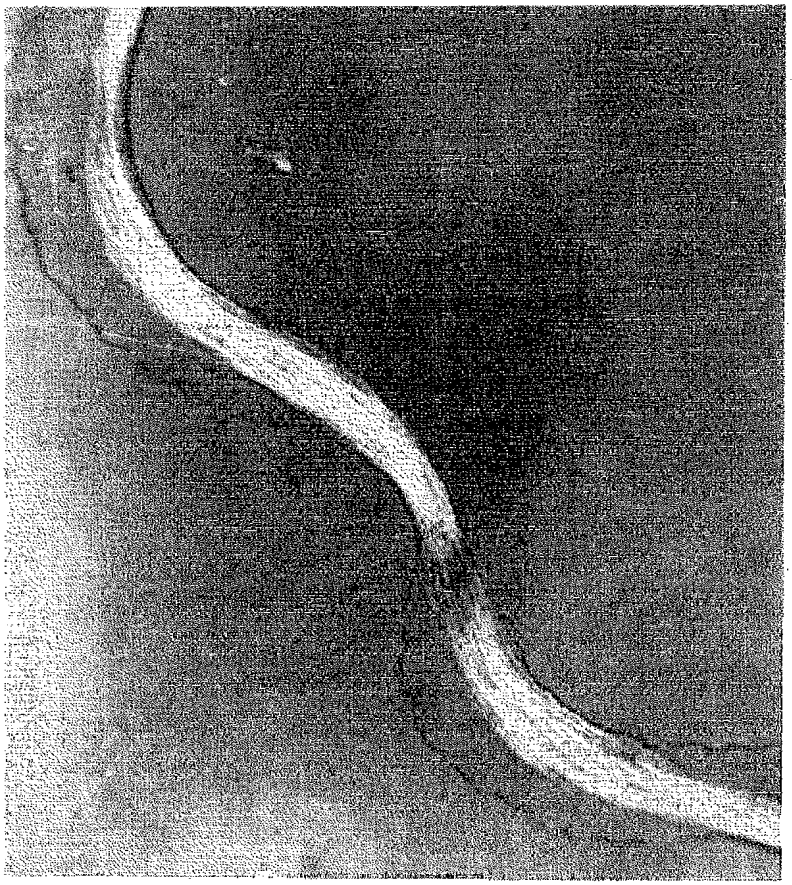


Fig. 2. Paracortex in wool. More resistant chemically, the paracortex remains fibrous and birefringent after 5 minutes exposure of the wool—a New Zealand medium-grade wool—to 6.5% aqueous sodium hydroxide at room temperature. The paracortex is seen at the inside of the fiber waves. (Photograph kindly furnished by J. Brant, The Toni Company, Chicago.)

This useful preliminary account is liable to some modification in detail as new evidence accumulates. In particular, Mercer (48) found that the fibrous structure of hair shows the α pattern and full orientation before the region of hardening, and that it can be disoriented, before hardening, without shrinkage. This makes it unlikely that contraction during keratinization can be due to protein chain folding into the α structure. An alternative mechanism for bending the fiber at the site of segmental keratinization may be available in the relative loss of turgidity in the more keratinized part.

An additional result that must be incorporated into the description is

the definite difference in properties of the two segments of the cortex of crimped fibers. It is inferred that keratinization is selective in the sense that the segment that hardens first tends to accumulate certain of the fiber components, such as sulfur, and perhaps to exclude other substances.

8. *Contributions of the Study of Keratinization to the Knowledge of Keratin Structure*

The main points of information that the microscopic study of keratinization contributes to the knowledge of keratin structure are the following:

Keratin is cellular. Even though keratinization is remarkably selective, in the sense of excluding nonprotein, the product is microscopically nonuniform.

Similarities in development of different keratin tissues suggests the presence of common structural components, in particular, the primary fibrous elements. The problem of nonuniformity of chemical composition is therefore resolved into two: namely, comparison of the composition of the various structural components of a single keratin and comparison of the same component of different keratins.

Hard keratinization is suggested to result from a special process or adaptation superposed on, or modifying, the soft keratinization mechanism.

The process of fibrillation, microscopic orientation (in distinction to the orientation within a fibril), and hardening occur successively but independently and to different degrees in the same structure.

The form of the surface scales of wool and hair and other microscopic structural features such as spindle cell orientation are accounted for. Plausible explanations, backed by a variety of experimental observations, have been advanced for medullation and crimp.

III. Properties of Solubilized Keratin Derivatives

1. *The Relation of Solubilized Derivatives to Keratin Structures*

This section summarizes relatively recent physical and chemical characterization of solubilized derivatives of wool, hair, and feather. Inasmuch as the chemical composition of keratins suggests no chemical reason for insolubility in aqueous media at moderate pH's and temperatures, it is proposed that these structures consist of extensive combinations of potentially soluble units joined with one another by at least two and often several chemical bonds. Because of differences in chemical nature and environment, these bonds may be expected to show varying degrees of specificity and reaction with available bond-breaking reagents and conditions. Studies of molecular size, size distribution, composition, end-groups, and other properties of solubilized derivatives can be used to get informa-

tion about the nature, distribution, and reactivity of the various bonds and the size and uniformity of various intermediate polypeptide units. Figure 3 is presented as a highly simplified model with a few formal resemblances to the actual keratin structure. In the figure, the blocks represent polypeptide units that are resistant to a given set of solubilizing treatments. The letters represent chemical bonds that are broken more or less specifically by various reagents. For example, A may represent a peptide bond particularly liable to acid hydrolysis; B may represent a peptide bond sensitive to base; and C may represent a disulfide bond, susceptible to

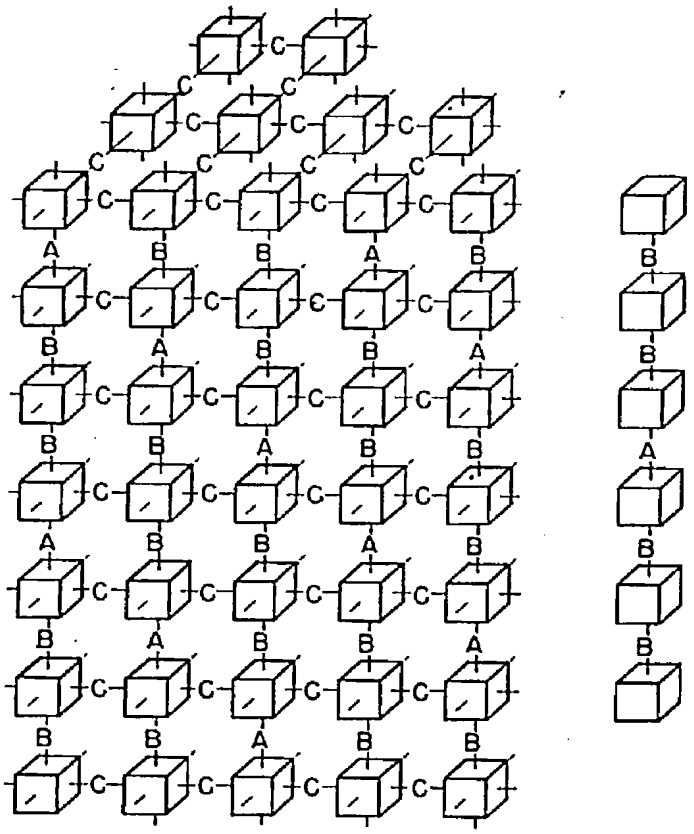


FIG. 3. Block diagram of keratin structure. The blocks represent polypeptide units, not necessarily identical, that are joined by various primary chemical bonds. *A*, *B*, and *C* represent three classes of such bonds. These are supposed to have different specific stabilities toward different solubilizing reagents. For example, *C* may represent the disulfide bond, specifically cleaved by certain reducing and oxidizing reagents. A solubilized unit released by the action of such reagents is shown at the right.

cleavage by oxidation and reduction, and also by hydrolysis under some conditions.

A reagent cleaving only C bonds will obviously release an intermediate unit containing six blocks which may be considered a fundamental polypeptide component of this model because no peptide bonds need to be broken to release it. Additional cleavage of A bonds, B bonds, or both results in successively smaller derivatives. It is evident at once that the model can be refined in several ways. However, it shows clearly how units of different size can be derived from a single structure. Since it is apparent that the solubilizing processes can rarely be considered absolutely specific, that bonds of a given chemical type can show a range of susceptibility to cleavage, and that the parent structure is microscopically nonuniform, variations among results of various solubilizing procedures are readily understood to be important evidence of structural details.

2. *Prekeratin*

Before describing the properties of solubilized keratin derivatives it is appropriate to mention studies by Rudall (64) and others on soluble keratin precursors from cattle nose epidermis. Molecular kinetic studies (51) of extracts in aqueous urea showed the presence of several distinct molecular weight classes. The major component was the smallest one observed. The others were reduced to similar or identical material by bisulfite. Oxidation by ferricyanide or hydrogen peroxide had a similar effect. The reduced prekeratin showed a single electrophoretic boundary with an isoelectric point near pH 4.2. The standard sedimentation constant[1] 1.7 and diffusion constant 2.4 were used to estimate the molecular weight 60,000 and the unusually high molar frictional ratio 3.5. These results apply to the reduced material in urea (25 g. of urea in 100 ml. of water) and to the main component, 80% to 90% of the original extract. The high molar frictional ratio may reasonably be ascribed to an elongated molecular unit. If sufficient evidence of this sort can be found, we will be justified in concluding that fiber formation on this scale occurs by association of fibrous rather than globular molecular units (49).

3. *Keratins Dispersed by Disulfide Bond Cleavage*

a. General Considerations. The final hardening process in natural keratinization is closely related to the disappearance of sulfhydryl groups and the formation of disulfide. It is therefore reasonable to try to reverse the process by means of reducing agents (25). Reduced keratins are, however,

[1] Throughout this review sedimentation constants quoted are in Svedberg units, 10^{-13} sec., referred to the standard medium water at 20°C. Diffusion constants are in units of 10^{-7} cm.^{2} sec.$^{-1}$, also referred to water at 20°C.

subject to reoxidation. The reduced keratin can be stabilized by alkylating the freed sulfhydryl groups (26), preventing their further coupling. Such reduced or partly reduced keratins are still not generally soluble in dilute neutral aqueous solvents. The need for a solvent such as a concentrated aqueous urea to dissolve the natural keratin precursor in skin has already been alluded to. Solvents effective for dissolving reduced keratin include alkali and concentrated aqueous solutions of hydrotropic reagents such as urea, guanidine salts, thiocyanates, ionic detergents, amides, and phenols (36). The nature of these solvents indicates that hydrogen bonds must be broken between individual protein chains in order to dissolve them. It is noteworthy that formation of hydrogen bonds apparently produces insolubility of the prekeratin in dilute aqueous media even before appreciable disulfide formation.

Solubilization by reduction should not be considered too literally a reversal of keratinization. The final stages of hardening include also the development of affinity for basic and nitro dyes which is not obviously related to the disulfide bonds present.

The principal reducing agents known to be effective in solubilizing keratins include sulfide, sulfite, and thioglycolate ions, and mercaptoethanol (monothioglycol).

Marked differences in the quantities of different keratin materials dissolved in solvent media containing different reducing agents or hydrogen bond breaking components show clearly that keratins, even samples of similar material from different species, are not all identical. The evidence also shows that a single keratin, feather in particular, can readily be separated into markedly different fractions.

Alkali cyanides have also been used to solubilize keratins (25). They are in general less effective than the reducing agents already mentioned. Higher alkalinity is required. Possibly as a result of the higher alkalinity, sulfur is lost from the protein. The soluble products are similar in some respects to those produced by other methods (23). In addition to its known reaction cleaving cystine, cyanide has also been described as an effective reagent for converting (combined) cystine to (combined) lanthionine (15). In spite of a recent demonstration by Ripa (62) that cyanide-treated wool does in fact contain demonstrable free sulfhydryl groups, the facts that only a small fraction of wool is solubilized by this reagent under mildly alkaline conditions and that the residue is resistant to solution by methods usually effective support the conclusion that stable lanthionine cross-links are formed by this reagent.

Oxidizing agents were shown to assist solubilization of keratins somewhat before their possible action as disulfide bond cleaving agents was suggested. Reagents used for solubilization include peroxy acids (peracetic acid, per-

manganate), hydrogen peroxide, chlorine or bromine (or hypohalites), and chlorine dioxide. Combined cysteic acid is commonly formed in quantity from the combined cystine. The strongly acid sulfonic acid group of cysteic acid assists solubility of the product under very mildly alkaline conditions, so that the products are readily studied in dilute aqueous media. Oxidants are commonly less specific in their action than are reducing agents. Some sulfur may be oxidized to sulfuric acid. The amino acid residues of methionine and tryptophan are rapidly attacked by performic acid. Chlorine dioxide attacks tyrosine, tryptophan, histidine, and possibly other residues at appreciable rates. In these secondary reactions the molecular weight of the products is not necessarily affected very much. The residues known to be most readily attacked (apart from cystine) form a relatively small portion of keratins. They are not known to be attacked in such a way as to break the polypeptide chain. Nevertheless, in solubilization even under neutral conditions it is desirable to apply some objective measure of peptide bond cleavage. This is emphasized by results of Middlebrook (55), who found that the number of amino end-groups accessible to fluorodinitrobenzene was doubled by exposure of wool to urea and bisulfite for 24 hours at pH 5 and 40° C. The corresponding average molecular weights for the intact and urea-treated wools were 60,000 and 30,000. Therefore the possibility clearly exists that some peptide bonds in wool are exceptionally labile. This possibility should be taken into account in interpreting results of solubilization experiments.

b. Reduction of Wool in Alkaline Solution. Sulfide. The action of sulfides on keratins has been recognized for a long time and is of industrial interest. The principal mechanism may be written (25):

$$\text{P—S—S—P} + \text{Na}_2\text{S} \rightleftarrows 2\text{P—SH} + \text{Na}_2\text{S}_2.$$

P—S—S—P represents "protein." In this instance the alkaline medium is effective for hydrogen bond breaking, permitting solution. Jones and Mecham (35) describe the rates and amounts of dispersion of wool, feather, hoof, and hair keratins under various conditions of concentration and temperature. In the case of wool, 70% was dissolved in 0.1 *M* sodium sulfide in 3 hours at 30° C. Nearly all of this was precipitated by acid at pH 4.2. The dispersion by sulfide may, however, be complicated by hydrolysis or possibly by the existence of disulfide bonds cleaved at different rates as indicated by the decrease in acid-precipitable material and the change in the character of the precipitate when preparations are made with longer times of treatment or with higher sulfide concentrations.

Molecular properties of wool dispersed in sulfide have been reported by Olofsson and Gralén (59, 60). Solubilization was commonly carried out in 3.5 *N* sodium sulfide at pH 11 for two days at room temperature. The

amount dissolved was not stated but may be taken to include most of the wool except the epicuticle and resistant cortical elements (50).

The sedimentation constant of the dissolved material was 0.8 to 1.0. Fractions made by successive extraction or by partial precipitation from the extracts showed the same ultracentrifugal behavior. An 8-hour treatment at 3°C. and a five-day treatment at room temperature gave extracts with the same sedimentation constant. However, the sedimentation constant gradually decreased with continuing exposure to the reagent.

The measured apparent partial specific volume, 0.705 cm.^{3} per gram, is a little lower than the 0.73 estimated from the amino acid composition of whole wool (80).

The weight-average diffusion constant, 9, was very little dependent on time, temperature, or partial neutralization of the extract. The preceding results give the molecular weight 9000. Other values given range from 8000 to 9500 (52). The same data yield the molar frictional ratio 1.78. This is the ratio of the resistance to movement through the liquid offered by the solubilized keratin compared with the resistance of a spherical, anhydrous molecule of the same dry mass. In the present case the average molecular unit was interpreted as equivalent in sedimentation and diffusion to an unhydrated prolate ellipsoid of revolution 170 A. in length and 11 A. in diameter. This apparent axial ratio was roughly substantiated by measurement of intrinsic viscosity. Assuming the average residue weight 128, Olofsson and Gralén deduce a degree of folding of the polypeptide chain intermediate between those of the α and β configurations. Smaller average residue weights have been estimated by other workers. The value 115 with or without allowance for a moderate degree of solvation gives an estimated degree of folding somewhat nearer that of the α form.

Electrophoresis showed no separable components. However, the ultracentrifuge records showed a boundary spread, independent of protein concentration, measurably in excess of that due to diffusion. Also the weight-average diffusion constant was 1.16 times greater than the different average given by the height-area method. All averages coincide for a homogeneous substance. The preparation was not, therefore, homogeneous. The results were interpreted as equivalent in effect to a logarithmic molecular weight distribution with about 20% of the substance below 4000 and 30% above 10,000 in molecular weight.

After two months contact with the solvent, the wool protein was found to have sedimentation (0.55) and diffusion (13.0) constants indicating the lower molecular weight 3400. The molar frictional ratio was slightly reduced to 1.65. The simplest interpretation is that the peptide chain is gradually shortened by peptide bond hydrolysis. If the unhydrated prolate ellipsoid is again taken as a model, the average length is then 60% of

the original, whereas the average molecular weight is only 40%. The ellipsoid model therefore suggests a relative extension of the protein structure when it undergoes cleavage. Additional evidence to permit critical tests of various possible interpretations is highly desirable.

Thioglycolate. An excess of various soluble thiol compounds such as sodium thioglycolate (25) and mercaptoethanol (36) is able to reduce the disulfide bonds of proteins by virtue of the reaction:

$$P—S—S—P + 2R—SH \rightleftarrows 2\ P—SH + R—S—S—R.$$

The protein chains are represented by P and the soluble thiol by R—SH. The independent chains may be dissolved if the pH is at least mildly alkaline. In this solubilization, the main effect of the alkali is believed to be the cleavage of hydrogen bonds between protein chains. The possible role of hydrolysis has, however, not been subjected to critical study. Jones and Mecham (36) compared the dispersing power of sulfide, thioglycolate, and mercaptoethanol, all at pH 12.7, for a variety of keratins. None of these reagents was consistently the most effective. The keratins, too, varied widely in their ease of solubilization, feather keratin being consistently highly soluble. Apart from sulfide dispersions, no alkaline dispersion has been further characterized except in the case of wool reduced by 0.1 *M* thioglycolate. Alkalinity higher than pH 10 is necessary to solubilize an appreciable fraction. Gillespie and Lennox (23) recognized four components of such extracts by characteristic electrophoretic mobilities at pH 11. The most alkaline component, at least 20% of the wool, was isolated in electrophoretically single-boundaried form. Still another component was solubilized by further treatment, with 1 *M* potassium hydroxide, and characterized electrophoretically. The fractionation plan and principal results are shown in Fig. 4. Similar electrophoretic patterns were given by wool solubilized in alkaline cysteine or potassium sulfide. Under the test conditions these reagents were only half as effective as thioglycolate.

The dissolution process has been studied under the microscope (20). The portion of the cortex toward the outside of the original crimp contributes the more readily soluble components. At least some of the more readily soluble part is in the β configuration or readily transforms into it (19). The less readily dissolved part of the cortex retains its birefringence, evidence of orientation, to about pH 12.6. The protein then dissolved gives cast films which infrared spectroscopy shows to have a stable α configuration.

The residue clearly shows outlines of scales and cortical cells, suggesting the presence of resistant membranes as described also by Mercer (50).

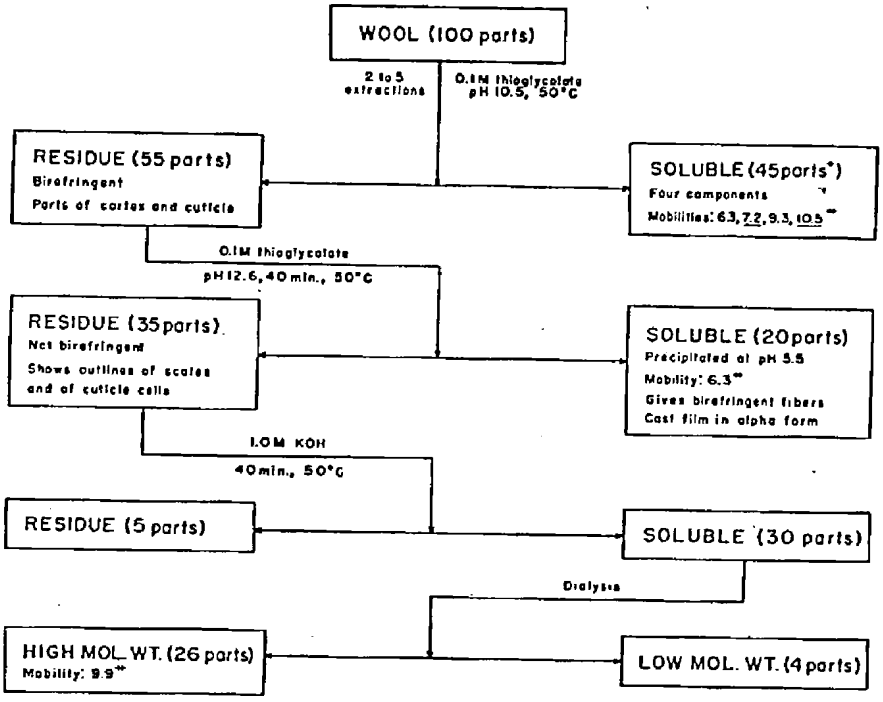
Cyanide. Cyanide has been classed as a reducing agent and tested for dis-

persing keratins (25) on the basis of its reaction with cystine:

$$\text{Cys—S—S—Cys} + \text{NaCN} + \text{H}_2\text{O} \rightleftharpoons \text{Cys—SH} + \text{Cys—SCN} + \text{NaOH}.$$

However, a higher alkalinity is necessary for solubilization or smaller amounts of keratin are dissolved than with thioglycolate or sulfide. For example, wool is mostly dissolved in a few hours in 0.5 *M* potassium cyanide in 0.1 *M* sodium hydroxide (25). Only 9 % is dissolved in 0.1 *M* potassium cyanide in 40 minutes at pH 12.6, 50°C. (23). The extracted material in the latter case showed a single, spreading electrophoretic boundary. On the other hand, Olofsson and Gralén (60) report that "no difference in action of thioglycolate, sodium sulfide, or sodium cyanide . . . could be observed." This statement may be presumed to apply to the approximate molecular weight of the dissolved material rather than to details of the dispersion and composition of the dispersed materials, for which differences are reliably reported.

Another distinguishing feature of the action of cyanide under the second condition cited is that the residue becomes resistant to solubilization with



*One 40 minute extraction solubilized 6%; two 20 minute extractions solubilized 25%.

**Times 10^{-5} cm^2 $volt^{-1}$ $second^{-1}$; Electrophoretic mobilities at pH 11, ionic strength 0.22, all negative.

FIG. 4. Fractionation of thioglycolate treated wool.

other reducing agents. With thioglycolate following cyanide, only 9% additional material was dissolved. This suggests the formation of lanthionine cross-links stable toward reduction (15).

c. Reduction of Wool in the Presence of Urea. Sulfite. A variety of protein denaturing agents is effective in replacing alkali for breaking hydrogen bonds of keratins. By the use of these agents it is hoped to avoid or minimize peptide bond hydrolysis, lanthionine formation, and other changes due to alkalinity. They may also be more effective than alkali. Wool is not dissolved at an appreciable rate in sodium sulfite at any pH. A considerable fraction is soluble in concentrated aqueous urea in the presence of sulfite. Molecular properties of dissolved fractions have been reported by Mercer and Olofsson (52), Olofsson (59), Woods (86), and Friend and O'Donnell (21).

Mercer and Olofsson solubilized about 20% of Lincoln wool with 5% sodium bisulfite in about 9.5 *M* urea at pH 8 by heating to 50°C. for 24 hours. The molecular properties were measured in 25% urea with 1% sodium bisulfite at pH 7. From the sedimentation (1.93) and weight-average diffusion (1.92) constants they computed the molecular weight 84,000 and the unusually high molar frictional ratio 3.86. Interpreted in terms of molecules approximating unhydrated prolate ellipsoids of revolution, these results correspond to an axial ratio of 90 to 1. Heterogeneity of the preparation is shown by the ratio, 1.19, of the weight-average diffusion constant to the height-area diffusion constant.

When the pH was raised to 12, the sedimentation constant fell to 0.94, practically identical with that observed for sodium sulfide dispersions. The change may be due to further reduction of disulfide bonds that were stable or inaccessible at lower pH; or the increased alkalinity may labilize other bonds of either primary or secondary valence.

Woods (86) was able to disperse about 30% of Merino wool by the method of Mercer and Olofsson (52) or by two days treatment at 50°C. with 4% sodium bisulfite in 8 *M* urea at pH 5.7. The intrinsic viscosities of these preparations fell in the range 0.1 to 0.2 deciliter per gram. The corresponding apparent axial ratio, for unsolvated prolate ellipsoids giving the same intrinsic viscosity, is in the range 10/1 to 16/1. Effects of pH and salt concentration were studied and found to be small. Conditions of dispersion and the urea concentration of the solvent used in the measurements had more important effects. The material solubilized at the higher pH had a distinctly higher intrinsic viscosity than that solubilized at the lower pH. The intrinsic viscosity increased regularly with urea concentration.

By osmotic pressure measurements, the number-average molecular weight of the wool dissolved at pH 5.7 was found to be 30,000. Taking into account diffusion of one-third of this material through the membrane,

Woods estimated the average molecular weight of the diffusible fraction to be 10,000 and that of the remainder, 50,000. Friend and O'Donnell observed values of 12,000 to 16,000 for similar preparations comprising 20% to 70% of the whole wool (21).

Mercaptoethanol. The relative effectiveness of the ionic disulfide cleaving reagent, sulfite, and the nonionic reagent mercaptoethanol is sensitively dependent on pH and other conditions of dispersion (36). Up to 60% of wool was solubilized in a single extraction with 0.5 *M* mercaptoethanol in the presence of 10 *M* urea and 0.2 *M* lithium chloride at pH 8 for 18 hours at 50°C. Extracts from wools of different breeds, geographical origin, and fineness showed similar properties (80). The average molecular weight 14,000 was computed from sedimentation and diffusion (5.0) constants measured at a protein concentration of about 2.5%. The corresponding molar frictional ratio was 2.8. However, if the limiting value of the sedimentation constant (1.57) for zero protein concentration is used with the observed diffusion constant, the dependence of which on concentration has not been reported, a molecular weight of 30,000 is obtained (21) with a molar frictional ratio of 2.15. This molecular weight is believed to represent an upper limit for the possible weight-average value for this fraction. Mercaptoethanol then appears to be more effective than sulfite in breaking disulfide bonds, since it gave, in quantity, solubilized material with lower molecular weight. It is possible that the two classes of reagents, nonionic and ionized (anionic), may react preferentially with different parts of the keratin structure. In this way either different portions of the total may be made soluble, or the same general part of the whole may be attacked but broken at different sites so that fragments of different sizes are formed.

d. Oxidation of Wool. Chlorine Dioxide. Solubilization of wool by aqueous chlorine dioxide, chlorine-free as a 1.25 *N* solution in the dark at 22°C., was introduced by Das and Speakman (16). The reagent does not attack the peptide bond of such peptides as leucylglycine. However, the treated wool undergoes a series of color changes that are believed to be related to the oxidation of tyrosine, as well as other changes in amino acid composition that may or may not be due to a difference in composition between the solubilized fraction and the residue. Most of the cystine is oxidized to cysteic acid. About 40% of the wool became soluble in the reagent in 120 hours. An additional 47% then dissolved in boiling water. The residue contained 1.3% sulfur as compared to 5.3% for the dissolved portion.

The molecular weight of the more readily soluble part of chlorine dioxide-treated wool was first reported to range from 2000 to 12,000 (71). The fraction of treated samples taken up in cuprietylene diamine gave the mean number-average molecular weight, by osmotic pressure measurements, of the part not diffusing through the membrance, of 13,000 (72).

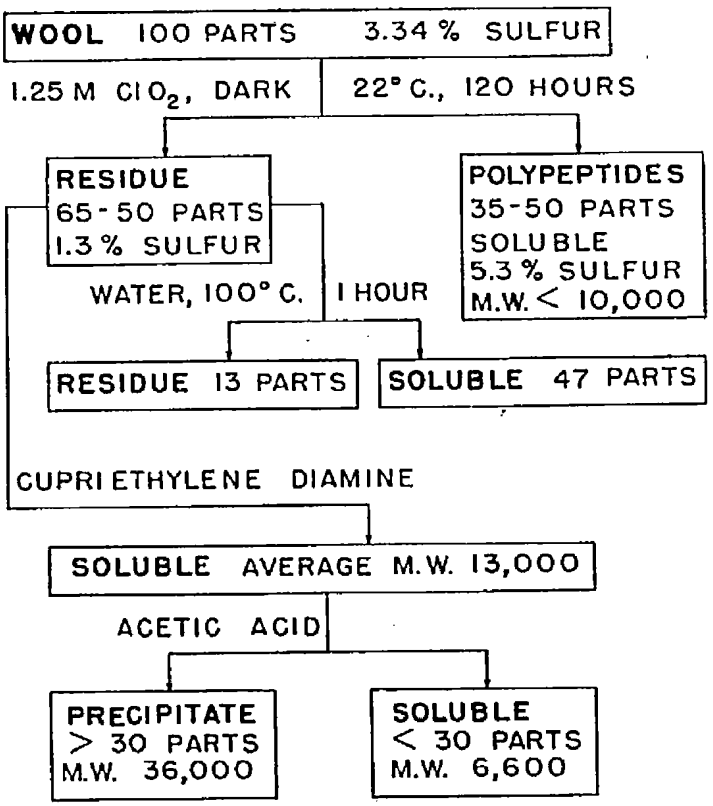


FIG. 5. Fractionation of chlorine dioxide treated wool.

Lincoln (coarse) and Merino (fine) wool and porcupine quill had this same value. Human hair gave 15,000. The oxidized Lincoln wool was separated by acid precipitation into a main fraction of average molecular weight 35,800 and a smaller alcohol precipitable fraction of molecular weight 6600. These relationships are shown in Fig. 5.

The viscosity of chlorine dioxide-treated silk in cupriethylene diamine is approximately half of that of untreated silk. Cystine is not present in silk. It is therefore inferred that chlorine dioxide does in fact labilize certain peptide bonds, possibly those adjoining tyrosine or proline residues.

Peracetic Acid. Hydrogen peroxide in acid media reacts reversibly with many acids to form peroxy acids. Such mixtures have been known for many years to have solubilizing effects on keratins (74). The action of solutions of peracids appears both more effective and more specific than that of hydrogen peroxide at neutral or higher pH (77). Peptide bonds are not significantly attacked under suitably controlled conditions, so that the

reagent performic acid has been used successfully to separate the individual peptide chains of insulin by oxidizing the disulfide links (65).

Alexander and his colleagues (1) have adapted these methods to wool solubilization. The insolubility and compact form of this material make much longer periods of treatment necessary. For example, 24 hours at room temperature in a 1.6% solution of peracetic acid were necessary for substantially complete reaction of the cystine. Methionine and tryptophan were also destroyed in accordance with their known reactivity. Peptide bonds are considered to be intact, but experimental study of this problem has not been reported. Little or none of the wool dissolved during treatment, but 90% to 92% was then soluble in 0.1 *N* ammonium hydroxide. The fractionation of such a preparation and the properties of the fractions are indicated in Fig. 6.

An α-keratose making up 50% to 60% of the original wool is precipitable from the ammonia extract by acidifying to below pH 3. This fraction contained 1.6% sulfur. It was prepared in an electrophoretically homogeneous form with a molecular weight of 70,000. The molecules were found to be spherical in dilute aqueous solution and precipitable in the α form The soluble α form was readily changed into a much less soluble β form. by short exposure to wet or dry heat or by evaporating a solution in dry

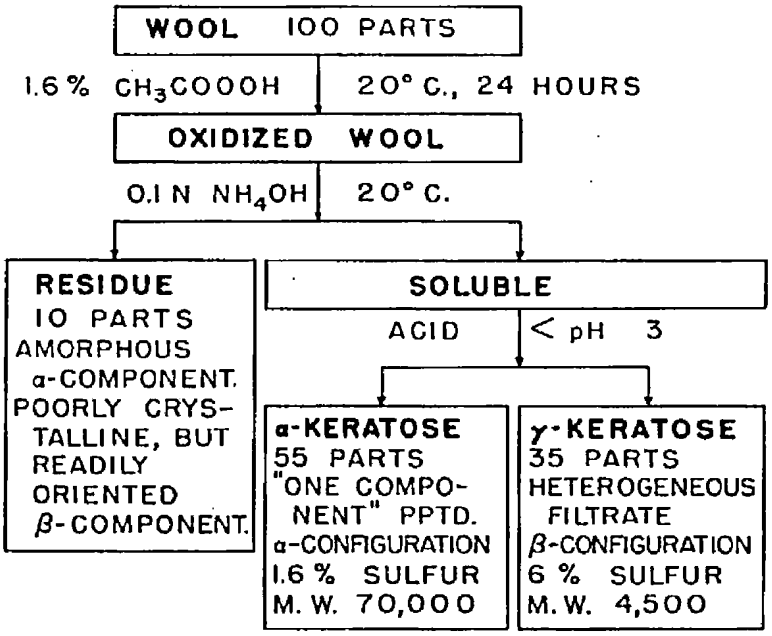


FIG. 6. Fractionation of peracetic acid oxidized wool.

formic acid. In this state the molecules are considered rodlike, with pronounced asymmetry.

About 35% of the original wool was not precipitated by acid. This γ-keratose fraction was polydisperse, with a molecular weight of about 4500. It contained 6% sulfur. It was recovered only in the β form.

The insoluble residue, 8% to 10% of the original wool, is of controversial origin (1, 30). The evidence suggests that it, like other insoluble residues from solubilization treatments, consists of resistant parts of the cortex and (mainly) cuticle, including epicuticle (50). It was first observed in the β configuration. Recent observations (31) of X-ray diffraction and infrared absorption indicate that the residue as isolated after treatment at 20°C. with minimum mechanical stress consists mainly of noncrystalline material in the α configuration together with an unoriented, poorly crystalline component in the β form. Heating and, in particular, slight mechanical stress markedly increase the crystallinity of the β phase and may produce orientation. On the other hand, films and oriented fibers could be prepared from the soluble fraction showing only the α form.

4. *Conclusions Regarding Wool Structure*

From the preceding results it is apparent that wool can be broken down into units of practically any desired average molecular weight. The next obvious step is to determine end-groups and disulfide in the various products to prove the relative roles of hydrolysis and disulfide cleavage. Our interpretation of the existing evidence is that the disulfide bonds of wool must be substantially completely broken before an appreciable amount of high molecular weight polypeptide material is dispersed. The largest fragments that can be solubilized have an average molecular weight of about 70,000 and contain a few intact disulfide bonds or their equivalent joining two or more polypeptide chains. Excluding the possibility of cyclic peptides or peptides with concealed end-groups, the average chain weight of the intact wool may be as high as 60,000. If so, the chains are hydrolyzed under exceedingly mild conditions to fragments of about 30,000 average molecular weight. Hydrolysis almost certainly occurs under alkaline conditions, permitting isolation of substantial quantities of material of molecular weight of 8000 to 10,000, and less. Nevertheless, some material of quite low molecular weight is formed together with the high molecular weight fraction. It remains to be decided whether this is a hydrolysis product of larger chains or whether it represents a distinct fiber component such as "cementing material."

In regard to molecular shape it is noteworthy that solubilized wool fractions almost always show unusually high molar frictional ratios. These have almost uniformly been interpreted reasonably, but inconclusively, as

evidence of elongated shape. By ignoring possible solvation or the effect of a randomly coiled, flexible molecule, it is possible to estimate as limiting values the axial ratios of rigid prolate ellipsoids having the observed hydrodynamic properties. Olofsson (59) has done this for several systems in which the dispersed wool had molecular weights from 8000 to 84,000. From these and similar results it is evident that the dissolved polypeptides do not exist as fully extended chains, but with a substantial degree of folding. This indicates either that some of the α structure is maintained in solution or that there is an approach to random coiling under thermal influences. Maintenance of α structure would require that a high proportion of hydrogen bonds giving configurational stability must remain intact even in supposedly effective hydrogen bond breaking media or that the α structure forms spontaneously when the protein is concentrated. It is of interest that fibers and films have been recovered in the α configuration from several dispersions. Coincidentally the estimated equatorial diameters of dispersions of molecular weight 8000 to 42,000 in three different media were near 11 A., approximately the value to be expected for the α configuration according to the Pauling-Corey model. On the other hand, wool is highly swollen in many of these solvents. The actual molecular asymmetry is therefore somewhat less than the limiting value and in fact has been found to be negligible in some cases. Another circumstance making it unlikely that dissolved wool has the approximate structure of a single extended cylinder in the α configuration is the existence of proline residues producing discontinuities, on the average, once in every 20 amino acid residues or 2000 molecular weight units. We are accordingly faced with the task of discriminating between a segmented rod and a more slender, more flexible, and longer, but coiled chain. If the former model, or something like it, has an objective existence, it should be possible to convert it into the second form by an appropriate change in environment such as raising the temperature.

5. *Feather*

It is interesting to compare the molecular properties of solubilized feather protein with those of wool, because these substances differ markedly in chemical composition (29), in solubility (35, 36), and in molecular configuration. Wool is typical of the highly intra-chain hydrogen bonded α group and feather of the β group, insofar as the more crystalline parts of the structure are concerned.

White Leghorn chicken feathers were used for all studies reported here. About 63% was dissolved by boiling 16 gm. with 11 gm. of sodium alkyl (C_{12}–C_{14}) benzenesulfonate and 1 gm. sodium bisulfite per 100 ml. The dried product had a protein-detergent ration of 1.1. Various molecular properties were measured in chloride-veronal buffer at pH 7.7 (84). Heter-

ogeneity was demonstrated in electrophoresis and diffusion experiments. The number-average molecular weight of the keratin moiety of the complex was 34,000. The corresponding approximate weight-average molecular weight from sedimentation and diffusion, again subtracting the part due to detergent, was 40,000. Molecular asymmetry or solvation of the complex was small.

Feather differs from wool in having a large fraction, about 80%, of the reduced keratin soluble in hot aqueous alcohol. The same fraction is dissolved directly in 10 *M* urea with 0.1 *M* mercaptoethanol and 0.2 *M* lithium chloride. The osmotic pressure of both preparations measured in this solvent gave a molecular eight of 10,000 (81). This value was observed at 25° and 45°C., at pH 5 and 8. It was not highly concentration dependent. Similar values were observed with aqueous veronal buffer at pH 8, without urea, using feather keratin made water soluble by treatment with chloroacetate after reduction.

Woodin (85) has further characterized feather keratin solubilized in urea by reduction with various reagents, or by subsequent oxidation with performic acid. The latter preparation had a single electrophoretic boundary. The various preparations were characterized by their cystine contents. In all cases the limiting osmotic pressure at zero protein concentration gave molecular weights in the range 9500 to 10,200. Similarly standard sedimentation constants (1.0) and intrinsic viscosities (0.15 deciliter per gram) varied little from preparation to preparation. These values give the molecular weight 10,000 and molar frictional ratio 1.7 (as prolate ellipsoids of revolution). Turbidity measurements gave molecular weight values near 11,000.

These results indicate that feather material is far more precisely degraded than wool to fragments having a relatively small range in size. The units are comparable in size to those isolated from wool by reduction in dilute alkaline media.

On the basis of evidence (61) that sorption of water and urea on wool is small, Woodin infers solvation to be nil. Therefore, the molecular asymmetry is deduced to be considerable. The molecular properties are very much like those of wool degraded to a comparable molecular weight. The discussion given concerning the molecular shape of wool applies also to feather. The apparent molecular asymmetry should not be accepted uncritically.

IV. Analytical Characterization of Keratins

This section is mainly concerned with the realation of amino acid composition to the structure of fibrous keratins. In addition, the relation of composition and structure to keratin properties is discussed. Finally the

problem of defining *keratin* is reviewed with reference to the analytical results.

1. *Composition of Representative Keratin Tissues*

Analytical results of keratins, as well as other structural proteins sometimes classed with keratins, are included in the compilation of Block and Bolling (9). Tristram (78) has recently given a detailed account of the amino acid composition of proteins in general. These references summarize most of the basic information needed for comparison of the amino acid composition of keratins and other proteins. For convenient reference as a basis for discussion, results for wool, human hair, cattle horn, porcupine quill, chicken feather, and human skin (keratinized epidermis) are collected in Table I. This table gives the number of grams of nitrogen, amide nitrogen, sulfur, and the various amino acids derived from 100 g. of keratin protein. The highest and lowest values reported in recent years for representative material have been selected. These data have been compiled from the following sources: for wool, references 29, 42, 70a, 71, and 83; for hair, 6, 9, 29, 35, 38, 44, and 45; for horn, 7, 9, 29, and 35; for quill, 6, 7, and 9; for feather, 7, 9, 29, 35, and 83; and for skin (epidermis), 6, 8, 9, 17, and 88. The case of cystine in wool will be discussed later in detail.

The first four keratins of this table are predominantly hard keratins with crystalline portions showing the α configuration. Wool has been the most thoroughly studied. Human hair, like hair of other animals, is closely similar to wool in composition in most respects, as shown in the table. Cattle horn is a hard keratin in a different form; porcupine quill is an extreme type showing a degree of detail in its X-ray diffraction pattern unusual for a keratin. These are also sufficiently like wool in composition that it is difficult to decide in most cases whether differences shown are due to analytical error or to real differences in composition. Chicken feather is predominantly hard keratin having the crystalline portion in the β configuration. Possibly on this account or because it is from a different order of animal it shows a few distinct differences from the predominantly α-keratins in composition, as in solubility and structure. Finally, results are given for the cornified epidermis of human skin, representing soft α-keratin. Although its analysis is incomplete and less assured, epidermis is of special interest as a prototype of other keratins, which are formed from specialized anatomical structures derived from it. In the cases of human hair and epidermis certain values, in parentheses, have been cited for hair and epidermis of other species.

TABLE I

Composition of Representative Keratins

Keratin	Wool	Hair	Horn	Quill	Feather	Epidermis
Source	Sheep	Human	Cattle	Porcupine	Chicken	Human
Type[a]	Hard	Hard	Hard	Hard	Hard	Soft
Structure[a]	α	α	α	α	β	α
Component	*Grams of component from 100 g. of dry keratin*					
Nitrogen, total	16.2 –16.9	15.5 –16.9	14.8 –16.9	15.8	15.0–16.2	14.2–15.5
Amide nitrogen	1.10– 1.37	1.17	1.14		1.09	1.16
Sulfur	3.0 – 4.0	5.00– 5.24	3.77– 3.9	3.0	2.9	1.9
Amino acids[a]						
With hydrocarbon side chains						
Glycine	5.2 – 6.5	4.1 – 4.2	9.6	5.7	7.2	6.0–(13.8)
Alanine	3.4 – 4.4	2.8	2.5		5.4	
Valine	5.0 – 5.9	5.5 –(5.9)	5.3 – 5.5		8.3– 8.8	4.2–(5.6)
Leucine	7.6 – 8.1	6.4 –(8.3)	7.6 – 8.3		7.4– 8.0	(8.3)
Isoleucine	3.1 – 4.5	(4.7)– 4.8	4.3 – 4.8		5.3– 6.0	(6.8)
Phenylalanine	3.4 – 4.0	2.4 – 3.6	3.2 – 4.0	3.6	4.7– 5.3	2.8
Proline	5.3 – 8.1	4.3 –(9.6)	8.2		8.8-10.0	3.2
Hydroxy						
Serine	7.2 – 9.5	7.4 –10.6	6.8	6.1–6.2	10.2–14	16.5
Threonine	6.6 – 6.7	7.0 – 8.5	6.1	3.9–5.4	4.4– 4.8	3.4
Tyrosine	4.0 – 6.4	2.2 – 3.0	3.7 – 5.6	3.3	2.0– 2.2	3.4– 5.7
Acid (free and as amide)						
Aspartic acid	6.4 – 7.3	3.9 – 7.7	7.7 – 7.9	8.7	5.8– 7.5	(6.4– 8.1)
Glutamic acid	13.1 –16.0	13.6 –14.2	13.8	17.6	9.0– 9.7	(9.1–15.4)
Basic						
Arginine	9.2 –10.6	8.9 –10.8	6.8 –10.7	7.6–8.0	6.5– 7.5	5.9–11.7
Lysine	2.8 – 3.3	1.9 – 3.1	2.4 – 3.6	2.6	1.0– 1.7	3.1– 6.9
Hydroxylysine	0.2	0				
Histidine	0.7 – 1.1	0.6 – 1.2	0.6 – 1.0	0.6	0.3– 0.7	0.6– 1.8
Heterocyclic						
Tryptophan	1.8 – 2.1	0.4 – 1.3	0.7 – 1.4	0.9	0.7	0.5– 1.8
Sulfur-containing						
Cystine	11.0 –13.7	16.6 –18.0	10.5 –15.7	8.0–9.5	6.8– 8.2	2.3– 3.8
Methionine	0.5 – 0.7	0.7 – 1.0	0.5 – 2.2	0.8	0.4– 0.5	1.0– 2.5
(Cysteine)[a]	0.4	0.5 – 0.8	0.8 – 1.6		0.4	

[a] Type and structure are given for the principal component(s). The amino acid composition is given in terms of free acids found in hydrolyzates. Cysteine is formed as an artifact in acid hydrolysis as described in the text. An effort has been made to give highest and lowest recent and valid values for composite samples. In the case of epidermis, the figures in parentheses are quoted without reference to the source of the keratin. In the case of hair, figures in parentheses refer to swine hair.

2. *Analyses in Terms of Amino Acid Residues and Functional Groups*

At least four properties of amino acid residues are significantly related to protein structure. First is the frequency of occurrence of residues, notably those of cystine, that can bind neighboring chains together with primary valence bonds. Second is the presence or absence of "polar" groups that can participate in forming hydrogen bonds between peptide chains or with water or other reagents. Third is the frequency of occurrence of residues of the amino acids, proline and hydroxyproline, which produce discontinuities in the direction of the peptide chains. (Hydroxyproline is not found

in keratins.) Finally, there is the bulk of the side chain contributed by a given residue. This is conveniently evaluated in terms of the residue weight. In addition, individual amino acid residues have in some cases been shown to affect the stability of a peptide chain toward hydrolysis.

To assist visualization of amino acid residues as structural components of polypeptide chains of keratin materials and as contributing chemically reactive groups of atoms, data from Table I have been used to derive Tables II and III. The side-chain structure contributed by each amino acid residue is indicated in Table II together with the frequency with which each of these residues occurs per hundred residues of the keratin protein(s). Cystine is given, as usual, as "half-cystine," because each cystine residue contributes two peptide units, —NHCHRCO—, either to different peptide chains or to different parts of the same chain.

Table III summarizes the analytical results in terms of the different kinds of reactive groups, or functional groups. These are expressed in gram equivalents per 10^5 g. of keratin protein. Mean residue weights have been computed from the nitrogen contents reduced by the amounts of nitrogen not taking part in the peptide bonds: these are compared with the mean residue weights given by the amounts of the various residues actually accounted for. An indication of the completeness and accuracy of these analyses, which leave room for further progress, is given by the percentages of the total weight, nitrogen, and sulfur accounted for in the quoted results. It is tempting to consider the higher results the more accurate because their sum often appears to account rather well for the total keratin substance. This inference should not be accepted uncritically. It will be noticed, for example, that the mean residue weight of wool computed from the reported amino acids differs somewhat from that computed from the nitrogen content and the amide, tryptophan, and basic amino acids.

3. *Specific Features of Keratin Composition*

In an authoritative review already cited (78) Tristram concludes, with reference to keratins and silk fibroin: "No particular amino acid distribution characterizes the biosynthesis of natural fibers." He thus recognizes the marked differences between silk and the keratins shown by their natural origins, composition, structure, and other properties. In the case of silk, the unusually fully extended, oriented β configuration with extensive hydrogen bonding between peptide groups of neighboring polypeptide chains is clearly related to the amino acid composition. The high proportion of amino acid residues with very small side chains (low residue weights) permits the necessary close fit required in the crystalline portions of silk. Keratin structure is more complicated. Keratins occur in both α and β

forms with less crystallinity than silk. However, they also show characteristic features of composition.

The high cystine content of keratins is widely, though not universally, agreed to be their most significant analytical feature. Skin (epidermis), which has the lowest cystine content of keratins, has more of this residue than most proteins. Feather protein has more cystine than three-fourths of the proteins in Tristram's compilation (78). Half-cystine forms one of every 6 to 12 amino acid residues in hard α-keratins. These are known to be exceeded in cystine content only by the hormones prolactin and insulin. Keratins show two features that distinguish them from these hormones. One is that the average polypeptide chain length of keratins so far examined is much higher than those of the hormones. The second is that the solubility of the hormones is not restricted by their disulfide bonds, whereas the insolubility of keratins depends directly on these bonds to a considerable extent. From this and related evidence it is inferred that the peptide chains of keratins are extensively cross-linked by disulfide bonds. Cross-linking of the peptide chains of the soluble high cystine proteins, on the other hand, is limited to specific combinations of relatively few chains.

Because of its high cystine content, the reversible fiber forming properties of insulin are of special interest. Insulin does not undergo any deep-seated change in structure when it becomes fibrous (37). However, the stability of insulin fibers is not controlled by the formation or destruction of disulfide bonds. Fibrous insulin is therefore fundamentally different from keratin in this respect.

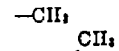
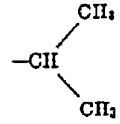
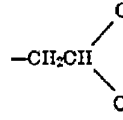
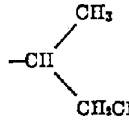
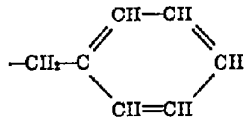
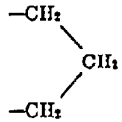
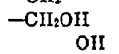
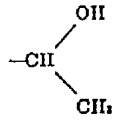
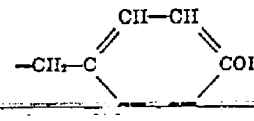
Even though keratins are characteristically stabilized by disulfide bonds, the range in their cystine contents makes it difficult to define *keratin* simply in terms of cystine content alone. Furthermore, some keratin samples or fractions, notably the medulla of coarse wool, have been found to have very little cystine, although these are also resistant to solubilization. In addition, keratins have been shown to become fibrous and to some degree resistant to solution even before they are stabilized by cross-linking. We conclude that disulfide cross-linking adds definite characteristic features of mechanical and chemical stability to keratins, but that other factors also govern keratin properties, for example, in the initial fiber formation. It is possible that the polarized aggregation of insulin is an applicable model for the initial fiber formation in keratins.

Small amounts of cysteine have been observed in acid hydrolyzates of keratins, as recorded in Table I. These are formed from cystine, partly by hydrolysis of the disulfide bond and partly by reaction with tryptophan (58). Free thiol cannot be detected in fully hardened keratin.

In addition to higher than average cystine contents, keratins all have high proportions of amino acid residues with other polar groups. These

TABLE II

Keratin Composition in Terms of Amino Acid Residues

Keratin	Wool	Hair	Horn	Quill	Feather	Epidermis	
Mean residue weight	113.9–113.9	118–112	120–112	113–114	116–107	126–135	
Residue	*Number per hundred residues*						*Side-chain structure*
Glycine	7.8–9.9	6.4–6.3	15.4–14.3	8.6–8.6	11.1–10.3	10.0–23.9	No side chain
Alanine	4.4–5.6	3.7–3.5	3.4–3.1		7.0–6.5		
Valine	4.8–5.7	5.5–5.6	5.4–5.2		8.2–8.1	4.5–6.4	
Leucine	6.6–7.0	5.8–7.1	7.0–7.1		6.6–6.6	8.0–8.5	
Isoleucine	2.7–3.9	4.2–4.1	3.9–4.1		4.7–4.9	6.7–7.0	
Phenylalanine	2.3–2.8	1.7–2.4	2.3–2.7	2.5–2.5	3.3–3.4	2.1–2.3	
Proline	5.2–8.0	4.4–9.3	8.0–7.9		8.9–9.3	3.5–3.7	
Serine	7.8–10.3	8.3–11.3	7.8–7.2	6.6–6.7	11.3–14.3	19.7–21.1	
Threonine	6.3–6.4	6.9–8.0	6.2–5.7	3.7–5.2	4.3–4.3	3.6–3.8	
Tyrosine	2.5–4.1	1.4–1.9	2.5–3.4	2.1–2.1	1.3–1.3	2.4–4.1	

Serine	7.8–10.3	8.3–11.3	7.8– 7.2	6.6– 6.7	11.3–14.3	19.7–21.1	—CH₂OH OH —CH CH₃
Threonine	6.3– 6.4	0.9– 8.0	6.2– 5.7	3.7– 5.2	4.3– 4.3	3.8– 3.8	
Tyrosine	2.5– 4.1	1.4– 1.9	2.5– 3.4	2.1– 2.1	1.3– 1.3	2.4– 4.1	—CH₂—C(CH=CH—C(COH)=CH)=CH	
Aspartic acid	5.5– 0.2	3.5– 0.5	7.0– 6.6	7.4– 7.4	5.1– 6.0	6.0– 8.2	—CH₂COOH
Glutamic acid	10.1–12.4	10.9–10.8	11.3–10.5	13.5–13.6	7.1– 7.1	7.8–14.1	—CH₂CH₂COOH
(Amide)	9.0–11.1	9.9– 9.3	9.8– 9.1		9.0– 8.4	10.4–11.1	- - - CONH₂
Arginine	6.0– 6.9	6.0– 6.9	4.7– 6.8	4.9– 5.2	4.3– 4.6	4.3– 9.0	—CH₂CH₂CH₂NHC(=NH)—NH₂
Lysine	2.2– 2.6	1.5– 2.4	2.0– 2.7	2.0– 2.0	0.8– 1.2	2.0– 6.3	—CH₂CH₂CH₂CH₂NH₂ OH —CH₂—CH₂CH CH₂NH₂
Hydroxylysine	0.1– 0.1						
Histidine	0.5– 0.8	0.5– 0.9	0.5– 0.7	0.4– 0.4	0.2– 0.5	0.5– 0.8	—CH₂—C(N=CH—NH—CH)=C	
Tryptophan	1.0– 1.0	0.2– 0.7	0.4– 0.8	0.5– 0.5	0.4– 0.4	0.9– 1.2	—CH₂—C(CH=CH—C(CH=CH—NH—CH)=C)=CH	
(Half)-cystine	10.4–13.0	10.3–16.8	12.1–14.6	7.5– 9.0	6.0– 7.3	2.4– 4.3	—CH₂S—
Methionine	0.4– 0.5	0.6– 0.7	0.4– 1.6	0.6– 0.6	0.3– 0.4	1.0– 2.3	—CH₂CH₂SCH₃
Constituents accounted for:			*Per cent*				
Total residues	87–107	89–106	101–105	60–64	92– 97	80–127	
Weight	76– 94	80–102	87–101	60–63	79– 92	71–100	
Nitrogen	83–101	91–104	99–104	62–65	85– 91	80–112	
Sulfur	82–101	92– 98	77–120	77–90	60– 79	70–144	
Mean residue weight as accounted for:							
	108	107–108	104–107	112	101–103	105	

TABLE III

Keratins as Chemically Reactive Substances

Keratin	Wool	Hair	Horn	Quill	Feather	Epidermis
Reactive group	*Gram equivalents per 10^5 g. of keratin*					
Free carboxyl	58–66	38–70	71–72		27–44	27–83
Amide	79–98	84	81		78	83
Carboxyl plus amide	137–164	122–154	152–153	185	105–122	110–166
Phenolic hydroxyl	22–36	12–17	20–31	18	11–12	19–32
Aliphatic hydroxyl	124–148	129–172	116	91–104	134–174	186
Total basic	78–92	68–91	59–92	65–68	46–59	59–120
Amino	20–24	13–21	16–25	18	7–12	21–47
Aromatic nuclei[a]	27–43	16–24	24–37	22	13–14	23–38
Half-disulfide	92–114	138–150	101–131	67–80	57–68	19–32
Oxidizable[b]	526–650	722–814	538–754	381–444	309–376	207–280

[a] Aromatic nuclei available for coupling with diazonium salts: tyrosine plus histidine.

[b] Oxidation capacity calculated as twice the number of oxygen atoms rapidly consumed from performic acid (Toennies and Homiller 77).

include, in the samples analyzed, unusually high proportions of the hydroxy amino acids serine and threonine. These constitute one of every six or or seven residues of the hard keratins. Silk fibroin and collagens exceed keratins in their contents of these residues. However, other fibrous proteins—fibrinogen, myosin, and elastin, and indeed most proteins—have much smaller amounts. Notable exceptions are Bence-Jones protein, γ-globulin, and several enzymes and other biologically active proteins.

The properties contributed to keratins by the hydroxy amino acids are less apparent than those due to cystine. These residues are able to take part in hydrogen bonding between chains, and theoretically they may also cross-link by ester formation with acid residues. This possibility has been mentioned from time to time but has so far escaped conclusive test. It is possible that serine is a precursor of cystine in the fiber. These properties would favor keratin stability. On the other hand, it is not apparent why these and other polar groups should not decrease fiber stability by favoring water uptake and solubility. It is known in addition that serine and threonine form peptide linkages that are specially susceptible to acid hydrolysis. For these reasons their presence in keratins would seem to be a factor decreasing stability.

The polar residues in keratins include substantial amounts of both acid and basic groups. Over half of the potential acid groups are found to be amidized, so that the actual free acid appears from analytical evidence to be slightly less than equivalent to the basic groups present. Although the total amounts are not unusually high for proteins (they are below the mean value shown in Tristram's compilation), they deserve attention because

changes in the balance of acid-base properties occur in the course of keratin formation. The protein loses its relative affinity for acid dyes and develops a preference for basic dyes as it hardens. Das and Speakman (16) have shown that the acid-base balance affects the water uptake of wool protein. Introduction of acid groups reduced the regain very strikingly. This decrease in water uptake is contrary to what one might expect from the addition of new ionizable groups. To account for such a result the new anionic groups may be suggested to form stable salt linkages with suitably accessible cationic groups in a way such that the ions are no longer available for water binding. Such a mechanism may possibly operate in natural keratinization.

Because of the variability of composition of keratins with respect to cystine, and because of the apparent absence of other constant and characteristic features of composition, Block and Vickery (10) were led to suggest that the basic amino acids histidine, lysine, and arginine occur in keratins in the characteristic molar proportion 1/4/12. Additional analytical results make it evident that this relation is only approximate and not constant. Many keratin materials do not show such a high relative proportion of arginine. These, Block (6) "tentatively named *pseudokeratins*" and proposed the definitive molar proportion (for histidine, lysine, and arginine) 1/3/3. The analytical proportions found for "*pseudokeratins*" actually ranged from 1/1/2 to 1/6/7. Even for typical hard keratins the ratio of lysine to histidine varies from 2.4 to 4.2 and the ratio of arginine to histidine, from 7.5 to 22.4 (9). A number of nonkeratin substances can be found with proportions of basic amino acids within these limits. Classification in terms of this proportion is therefore not absolute. However, the arginine content of keratins in general is higher than that of most proteins, whereas that of histidine is very low.

Hard keratins have very low contents of certain essential amino acids: histidine (as mentioned), methionine, and tryptophan. Feather, in particular, is also deficient in lysine. Skin keratin differs from the hard keratins in having a definitely higher content of methionine. These results suggest a certain economy in the use of these metabolically critical amino acids in the production of metabolically inactive keratin.

Hydroxylysine has been shown to occur in wool in an amount probably not over 0.18% (54). Its presence in wool has been confirmed, but its occurrence in human hair could not be shown definitely (34). Although hydroxylysine has been found in several proteins, it is not abundant. Its occurrence has received ample confirmation only for collagen or gelatin, which yield about 1%. The occurrence in wool of a measurable amount suggests the speculation that the wool fiber may include a polypeptide fraction that occurs also in collagen. The speculation is encouraged by the

close association of keratin with collagen-forming tissue. Against it are the clear differences between the X-ray diffraction patterns of collagen and wool and the absence from keratins of hydroxyproline, an amino acid occurring abundantly in collagens.

The configuration and manner of packing of polypeptide chains is known to be affected by the bulk of side chains present and by the occurrence of proline or hydroxyproline residues, which introduce local changes in direction of the polypeptide chains. The keratins appear to contain about twice as much proline as an "average" protein, although they have much less than the collagens. The average side-chain bulk is conveniently compared in terms of the mean residue weight. The value for wool is probably within a few per cent of 114. Table II shows that the hard keratins are equal in this respect. The value for feather is lower, but so little lower that the available data are not adequate to establish a real difference. The value for skin seems appreciably higher, 130, although the data have not been sufficiently checked to give much confidence in the exact value. It is evident that the keratins have higher mean residue weights than the other proteins occurring normally in the fibrous state.

The relationship of protein structure to these factors is readily summarized with reference to Fig. 7. This again shows the unique position of silk fibroin in its low proline content and low mean residue weight. These are undoubtedly related to its perfection of orientation with fully extended (β) polypeptide chains and its consequent strength and very limited elastic extensibility. Incorporation of 15% to 30% proline plus hydroxyproline, with an increase in residue weight to the approximate range 80 to 95, precludes formation of the fully extended structure but permits that characteristic of collagens, of which elastin may be regarded as a variant. These show moderate elastic extensibility when wet. Most of the other natural fibrous proteins, those belonging to Astbury's keratin-myosin-epidermin-fibrin (KMEF) groups, have a mean residue weight in the range 112 to 120, with the possible exception of epidermin, which is not sufficiently well characterized to be classified with certainty. In this group, the presence of the large side chains favors hydrogen bond formation between peptide groups of any single given chain rather than between peptide groups of different chains. This tendency produces the folding characteristic of α-proteins. Within this group, keratins, fibrin(ogen), and myosin can be differentiated on the basis of their proline contents, respectively, about 8%, 6%, and 2% and, better, their sulfur, respectively, 2.0% to 3%, 2.3%, and 1.4%. The group is characterized by long-range elasticity that is at least partly due to its lower degree of crystalline perfection.

Although the crystalline portions of fibers of the KMEF group occur usually in the α, folded state, it has been shown that the extended, β form

exists to an appreciable extent in the rest of the fiber, and that this fraction can be increased and oriented by mechanical stress. We may postulate that the balance between the α and β forms is controlled to some extent by the mean residue weight. Keratins like feather that occur naturally with crystalline portions in the β form would therefore be expected to have a lower mean residue weight than other keratins. This prediction is not convincingly supported by the data of Table II, which shows the mean residue

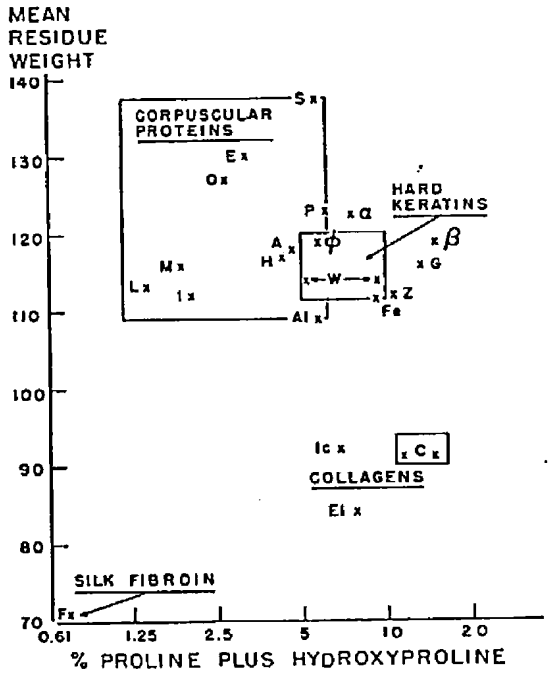


FIG. 7. Protein structure, residue weight, and proline content.

Key:

A	Serum albumin	L	Lysozyme
Al	Aldolase	M	Myosin
C	Collagens	O	Ovomucoid
E	Epidermis (Table I)	P	Prolactin
El	Elastin	S	Salmine
F	Silk fibroin	W	Wool (Table I)
Fe	Feather (Table I)	Z	Zein
G	Gliadin	α	α-casein
H	Edestin	β	β-Casein
I	Insulin	ϕ	Fibrinogen
Ic	Ichthyocol		

The range of values reported for hard keratins is enclosed in a rectangle. The range of reported proline contents for wool is shown by arrows. Similarly, ranges of values for a number of representative collagens and for nearly all corpuscular proteins are included in the respective rectangles.

weight of feather to be only slightly less than that of the hard α-keratins. The proline content of feather is only slightly higher than that of the hard keratins. The anatomy of feather formation does not suggest the occurrence of mechanical stresses different in order of magnitude from those acting on α keratins. Accordingly, if the distinction between the α and β forms is governed by the amino acid composition, it is likely that the arrangement of the residues, possibly with some segregation of components, is important in addition to mean residue weight. Otherwise it must be inferred that the balance of α and β configurations is very critically dependent on the balance of mean residue weight and disorientating factors.

4. *Distribution of Amino Acid Residues in Keratins*

An effort has been made in the preceding paragraphs to specify features of amino acid composition that are characteristic of keratins and to point out ways in which composition is related to keratin properties and structure. However, detailed understanding of protein properties almost uniformly requires information about the distribution of the various amino acid residues and finally their order and arrangement in the polypeptide chain. Although methods effectively used in discovering the amino acid sequences of beef insulin were initiated in the study of wool, determination of the detailed chemical structure of wool protein(s) has been slower because of their far greater complexity. Other keratins have not received comparable attention.

Two main lines of research have been begun to discover the arrangement of amino acid residues in wool. The first is the determination of which residues occur at the end of polypeptide chains. The second is the identification of small peptides in partial hydrolyzates, to determine which residues occur next to one another. Isolation of a given peptide has additional implications for keratin structure in that it requires stability of certain peptide bonds, and lability of others, toward the particular hydrolyzing conditions used. A prerequisite to substantial progress in both of these approaches is the isolation and characterization first of structural components and finally of individual proteins.

5. *End-Groups*

In 1949, Middlebrook (53) reported the identity of the amino acid residues occurring with free α amino groups at the ends of protein chains in wool. These were determined as the dinitrophenyl derivatives isolated after hydrolysis of Lincoln wool (coarse, relatively straight, and not medullated) treated under mild conditions with fluorodinitrobenzene. An admirably detailed report (55) gave carefully corrected quantitative estimates. These and comparable values (72) for Romney (medium) and Merino (fine,

TABLE IV

N-Terminal Amino Acid Residues of Wools and Hair

Observer	Middlebrook	Tibbs	Tibbs	Tibbs	Blackburn
Keratin:	Lincoln wool	Romney wool	Merino wool	Human hair	N. Z. 56's wool
Amino acid					
Glycine	5.2	4.5	4.7	3.9	0.5
Alanine	1.3	1.2	1.2	1.0	1.4
Valine	2.4	2.4	2.4	4.0	4.0
Serine	1.3	1.2	1.2	1.0	0.2
Threonine	4.8	4.9	4.9	4.0	2.2
Aspartic acid	0.6	0.6	0.6	0.5	0.3
Glutamic acid	1.3	1.2	1.2	1.0	0.8

The amounts of terminal amino acids with free alpha amino groups are given in gram equivalents per 10^6g. For references, see the text. Blackburn's figures have not been corrected for losses of the terminal amino acid derivatives during hydrolysis.

highly crimped) wool and human hair are given in Table IV. There are, in wool or hair, about 1 aspartic acid, 2 each of alanine, serine, and glutamic acid, 4 to 8 valine, 6 to 8 threonine, and 6 to 9 glycine gram-equivalents with free α-amino groups per million grams of keratin. Blackburn's values (5) for New Zealand 56's (medium-coarse) are included for comparison, although they have not been corrected for loss of DNP-acids during hydrolysis. It is possible (5) that comparable amounts of terminal cystine with free amino groups could have escaped detection because of the instability of dinitrophenyl-cystine.

A preliminary report (87) of application of hydrazinolysis to the determination of the amino acid residues with free carboxyl groups in wool cites these as serine, glycine, threonine, and alanine.

These results illustrate very well the natural heterogeneity of keratin. Of course the proportions could be greatly affected by the presence of a small amount of a low molecular weight polypeptide "impurity." However, it seems unlikely that wool samples cleaned with reasonable care would be contaminated regularly with any protein not a normal fiber component. The identity of the amino end groups has been independently confirmed. The proportions have been reported to be approximately the same for several different wool and hair samples. The general character of the results therefore seems assured in spite of substantial empirical corrections for hydrolytic loss of the DNP-derivatives. From the evidence given there are at least seven different kinds of polypeptide chains in wool

or hair. The most common of these may occur eight or ten times as often as the least abundant.

The total amounts of terminal residues estimated permit calculation of the number-average "molecular" weight of the polypeptide chains. These values range from 59,000 to 65,000 (the latter for hair). Comparison with values found by other methods with solubilized wool has already been made. It is of special interest that 24 hours exposure to 1% sodium bisulfite in saturated urea, pH 5, at 40°C., followed by precipitation with acid, washing, and drying, approximately doubled the quantity of end-groups found in both the dissolved and undissolved fractions of wool (55). The new end-groups were contributed by the same amino acids and in the same proportion as originally, except for terminal threonine, which was unchanged. Middlebrook observed that all of the ϵ-amino groups of lysine reacted with the fluorodinitrobenzene. He inferred accordingly that the wool was completely accessible to this reagent. We are therefore faced with the problem of deciding whether the accessibility of all lysine residues is an adequate test of the accessibility of all other parts of the solid keratin structure,[2] or whether the effect of urea under apparently mild conditions is really hydrolytic for some peptide bonds. Middlebrook (56) has also suggested that some non-peptide forming α-amino groups may assist in linking polypeptide chains in such a way, for example, by hydrogen bonding, that they are not free to react with fluorodinitrobenzene under the usual conditions until they have been released by certain treatments such as with alkali at pH 12.

The four amino acid residues with free carboxyl groups also occur with free amino groups. These and aspartic acid are distinguished by very short side chains—a circumstance that is associated with special lability of peptide bonds in which they participate. Glutamic acid is also often liberated early in hydrolysis. One therefore is led to wonder whether steric or other effects that make peptide bonds of these amino acids specially reactive with water also leave their free amino groups or carboxyl groups specially accessible to end-group reagents.

The most interesting feature of these end-group analyses is the suggestion of simple, whole number relationships among the residues. Such relationships are certainly permitted by the experimental variation indicated in Table IV, which may also result in part from uncertainty in empirical corrections for the large losses of some of the end-group derivatives during hydrolysis. However, the same latitude that allows the possibility of integral relationships also makes such an inference less compelling and permits the alternate, more likely interpretation that at least some of these

[2] The ϵ-amino groups of hydroxylysine appear to be not accessible (51)

polypeptide chains may vary in proportion independently, even though within a restricted range. Much evidence is against the concept that wool is essentially a homogeneous, though complex, substance—a thesis developed with remarkable ingenuity by the Leeds University group (55, 72). The most conclusive of such evidence is the repeated isolation of wool fractions corresponding to microscopically distinct components that differ markedly in composition from whole wool.

The data of Table IV also suggest that there may be a slight difference between wool and hair in average chain weight and in the proportion of end-groups, notably valine. The apparent analytical precision is not sufficient for a final judgment of identity or difference in this respect. However, some limits are indicated for the possible variation.

6. *Sequences*

A beginning has been made in the determination of amino acid sequences and groupings in wool, with the work of Martin, Consden, and their colleagues (12–14, 28, 47), in characterizing a large number of small peptides from partial acid hydrolyzates, and of Blackburn (5) on papain digests.

Blackburn (5) has described the action of papain on wool with respect to the amino end-groups and the composition of the soluble fraction. All fractions of the treated wool showed increases in end-groups, but leucine (or isoleucine) was the only new *N*-terminal residue different from those of the original wool. Soluble peptides averaging five residues in length constituted one fraction which was further fractionated by chromatgography. The constituent amino acids were identified, although separation into individual peptides was not achieved. The best characterized subfractions contained glycine, alanine, valine, and leucine, with or without lysine, cystine, glutamic and aspartic acids. They were remarkable in having little or no serine or threonine, although the fraction as a whole had substantial amounts of these as end-groups.

For reasons of experimental convenience, most amino acid sequences or groupings so far identified in wool have been those involving glutamic acid, aspartic acid, or cystine as a component, combined as dipeptides. As a result it is now known (12) that a considerable proportion of glutamic acid residues in wool occur next to one another, since glutamylglutamic acid was far more abundant than any other acid dipeptide in a partial acid hydrolyzate. About 10% of the glutamic acid was found to be combined in this way. In addition, aspartylglutamic acid, glutamylaspartic acid, and peptides of glutamic acid with tyrosine, serine, and threonine have been found in amounts comparable to those of dipeptides containing one polar and one nonpolar residue. Therefore, there is not a strict alternation of polar and nonpolar residues in wool.

No dipeptides containing two basic residues were found.

Certain amino acids, serine and alanine, occur more frequently next to glutamic or aspartic acids than to a basic residue. Others, valine and leucine, seem to be more equally distributed.

Cystine occurs in wool next to most other amino acids (13). Peptides containing cysteic acid (from cystine) combined with serine, alanine, threonine, and glycine were isolated in largest quantity, together with decreasing amounts of dipeptides with valine, leucine (or isoleucine), phenylalanine, aspartic acid, and glutamic acid. Tyrosine was not found next to cystine. The similar failure to find dipeptides including proline residues may be due to experimental difficulties. The presence of proline residues near or adjoining cystine in substantial amounts is asserted in several places to be a significant feature of wool structure (12, 13, 55).

7. *Variability of Keratin Composition*

The selection of examples for Table I was made to indicate the range of composition found for a given keratin material and to show differences and likenesses among various types. Block (7) has emphasized the unusual range of composition among keratins, in contrast with many groups of homologous protein tissues. Apart from analytical problems, variation is very likely due in part to changes in proportions of components. The main problem in keratin analysis may be the separation and purification of the anatomical components, and then their polypeptide subcomponents, in an understandable and reproducible manner.

Although analyses of whole keratin materials tend to complicate the interpretation of differences, some unequivocal differences in composition appear to be established among keratin types, among similar structures of different species, and among different structures of a single animal, and it has further been established that a single keratin material, wool, for instance, is not critically uniform in composition even as produced from a single follicle. These variations are of great interest. In addition to their practical importance in relation to wool quality, the variations are evidence of the conditions and processes of keratin formation.

8. *Analytical Distinction of Keratin Types*

Two kinds of judgment will be attempted here. The most apparent differences among the keratins of Table I will be mentioned. Second, some of the more obvious analytical inadequacies will be indicated.

Discrimination between hard and soft keratins resolves itself into comparison of skin (epidermis) and the specialized keratins. Skin is inadequately characterized in view of its fundamental importance, especially in that abnormal material has commonly been used and because it has not had

appropriate independent study. However, its protein has long been recognized to differ from the hard keratins in its low cystine content (24) and higher methionine, which may equal the cystine. Available data show it also unusually high in serine, relatively low in threonine, proline, and phenylalanine. Other important differences may be concealed in the range of values reported for glycine, glutamic acid, lysine, and arginine in particular. It is attractive to consider epidermis to be a primitive keratin, that is, less specialized than hair and the others. On this basis we may *speculate* that its composition is relatively little altered from that of an average body protein (whatever that is) so that any unusual features, such as the high serine, may be interpreted as fundamentally necessary for a keratin. On the same basis, one may surmise that incorporation of cystine cross-links in quantity in the more complex keratins is a secondary adaptation not biologically necessary to a keratin. This interpretation may be supported by the unusual reported variation in cystine content of the hard keratins. An additional secondary specialization may be the decreases observed in some of the nutritionally essential amino acids in the hard keratins.

Among hard keratins, feather shows distinctive features of composition that may be provisionally related to its assumption of the β structure in its crystalline portion. Feather is somewhat higher in valine and phenylalanine than the other keratins cited but distinctly lower in lysine. It has lower contents of cystine, glutamic acid, arginine, and tyrosine than the other hard keratins but appears to be higher in serine. Its slightly lower mean residue weight has been mentioned. Structurally the most complex of keratins, feather is readily separated into fractions (82) different in composition. The less readily solubilized part is lower in cystine and sulfur than the rest.

The group of hard keratins having crystalline regions with the α configuration shows, on the whole, surprisingly little variation from member to member, even though ranges of values reported for individual members call attention to need for further study. Porcupine quill, which is cited because of the special interest in its detailed X-ray diffraction pattern, is the most inadequately characterized material of the group. However, it appears to have less cystine, serine, and threonine but more glutamic acid than the others.

9. *Variability of Cystine*

Cystine, in spite of analytical difficulties in its determination, appears to show clear evidence of characteristic differences among the hard, α-type keratins, as among keratins in other comparisons. This variation is supported by differences in total sulfur, because the other sulfur-containing

residue is relatively constant and much smaller in amount. For illustration, data of Lindley (43) may be cited. These show a range in total sulfur from 3.3% to 5.3%, for hair of various animals and the low value 2.0% sulfur, for ram's horn. These differences are of special importance, as repeatedly suggested, because of the special role of cystine in stabilizing the keratin structure. Characteristic differences in mechanical and chemical properties can be anticipated as a result. Such differences in composition and properties appear also among samples of apparently identical character, even among different sections of the same structure. For example the hooves of cattle, swine, and horses show considerable variation in swelling in water and in rate of digestion by proteolytic enzymes (57). By some criteria the dissolved portion should not be considered keratin. However it contains sulfur and cystine, although less than the more resistant residue, and is not anatomically distinct. It should probably be regarded as incompletely consolidated keratin.

Wool shows some additional points of interest. As a result of careful comparisons by many workers over an extended period, the sulfur content of wools of various origins is known to vary at least from 3% to 4% (3). These figures are for macroscopic samples of what may be called "ordinary" wools. The lower values are commonly observed for the coarser, especially kempy*, wools. In these cases, low sulfur content is associated with the presence of a medulla, characterized as a soft keratin component with little or no sulfur (4).

The sulfur content is known to be in part under nutritional control. This may be due in some instances, with medium or coarse wools, to variation in the proportion of medulla (70). Medullation can be produced in some breeds of sheep by nutritional deficiency in methionine (46). Some of these kempy wools were found to have from 7.3% to as little as 3.5% cystine, in direct comparison with normal wools of 9.5% to 12.5% (referred to 16% nitrogen). The contents of methionine and lysine were normal. However even in a fiber with a large medulla, the latter forms only a small fraction of the total mass. It is therefore evident that the cortex, which forms most of the fiber, also is low in cystine in these cases. The cystine content is therefore not controlled simply through the proportion of medulla. Additional evidence of this is given by Bonsma (11), who found that the middle portions of Merino (non-medullated fine wool) fibers grown during the dry season on natural pasture were a few tenths of a per cent lower in sulfur than the root and tip ends, which were grown at a time of year when the nutritive value of the pasture was much higher. Sheep fed a uniform ration throughout the year did not show comparable seasonal variation. However, differences between the sulfur contents of fleece

* Kemp: Coarse hair in wool.

samples from different sheep were as great as those presumed to be due to the known nutritional variation. From these limited results it may be concluded tentatively that variation in sulfur and cystine content due to diet exists, but that ordinarily such variation is less than that due to individual differences among sheep.

Even wider variation is reported (4% to 1% sulfur) within a single lock of Lincoln wool (63). Similarly, estimates of the cystine contents of individual Cotswold and Buenos Aires fibers varied respectively from 3% to 15% (0.8% to 4% sulfur) and 7% to 18% (2% to 5% sulfur) (75). In the latter study, root halves were found to contain, on the average, 2% more cystine than the tip halves. Both Ripa and Speakman (63) and the workers at the Textile Research Institute (75) compared sulfur or cystine contents with mechanical properties. The results seem to establish that the behavior of fibers in reversible extension is governed by the hydrogen bonds between the protein chains, whereas the cystine cross-links affect the behavior of the fiber only when the stress is high enough to cause plastic deformation.

It has already been mentioned that these variations in sulfur content cannot be due entirely, or even largely, to variations in the degree of medullation, even though the medulla is usually lower in sulfur than the cortex, which forms the bulk of the fiber. A possible way in which such variations may result is through differences in proportions of fibrillar and cementing components of the cortex, that is, between relatively crystalline and relatively noncrystalline portions, supposing these to be intimately mixed. On the other hand, a mass of evidence has been accumulated and has recently been interpreted (19, 33, 50) as showing a clear division of the cortex in many fiber cross sections into segments differing in dye-affinity, swelling, supercontraction, enzyme digestibility, and solubility in alkaline reducing media. Differences in chemical composition were therefore practically assured. Mercer (50) has proposed a distinction between "keratinous" and "nonkeratinous" constituents. Spindle cells as isolated by Lindley (42), for example, by partial acid hydrolysis showed progressive concentration of cystine in the resistant *fraction* to the remarkably high value of 25%. This and similar evidence suggests that the more resistant cortical *segment* is characterized by a higher content of sulfur and cystine. The characterization is incomplete in that it has not yet been determined whether (a) the protein chains of the two segments are essentially similar in amounts and compositions except for differences in the frequency of disulfide cross-links in the one, whether (b) the segments differ in the proportions of protein chains of composition different in other respects, or whether (c), as an extreme case, one segment has a protein chain constituent quite different from any occurring in the other.

10. *Conclusions Regarding the Variation in Cystine Content*

The cystine content of hard, α-type keratins is probably the most significant, but yet the most variable, feature of their composition. In many other respects the amino acid contents appear rather similar throughout the group. Sulfur or cystine contents, however, discriminate species, individuals within a species, nutritional status, follicles on an individual, and almost certainly, in some instances, the two sides of a follicle. The evidence suggests that the keratin substance is formed and its disulfide cross-links built in by separate processes under independent or partly independent control. The cystine content would then depend on the balance between these processes.

11. *Composition of Protein Fractions or Components*

The last section mentioned differences in the distribution of sulfur or cystine among different parts of the wool fiber. A number of attempts have been made to analyze separate components in other respects. Some of the results are collected in Table V. In general, methods of fractionation use degradative procedures, so that it cannot be stated positively that the solubilized fractions do not include material originally part of the residual microscopic component. That is, the solubilized fractions, sometimes identified as cementing material of cortical origin, may represent (a) grossly detectable microscopic components such as the exocuticle or the less resistant cortical segment; (b) distinct polypeptide species intimately associated with other, more effectively crosslinked or more crystalline portions; (c) specially labile sections of more generally resistant polypeptide chains.

Table V
Composition of Components of Wool and Hair
(Grams per 100 g. of protein)

12. *Relation of Keratin Properties to Composition and Structure*

a. Solubility. Characteristic properties ascribed to keratins are commonly (a) insolubility in water, including aqueous solutions of salts, hydrotropic substances, and dilute acids and bases at temperatures not much above room temperature; (b) resistance to proteolytic enzymes; (c) resistance to hydrolysis. The insolubility of a typical keratin such as wool is readily verifiable by the absence of substances giving standard tests for proteins and amino acids from water extracts of the naturally occurring, greasy, keratins. After severe mechanical damage not more than 3 % of the protein became soluble in neutral or dilute alkaline solution (83). The amount of protein or protein degradation products observed in wool processing wastes is therefore a measure of damage preceding or during the processing, or of extraneous protein. It is necessary to discuss the basis of this insolubility in general elementary terms because the necessary theory and data are not precise enough to give an exact detailed account.

TABLE V

Composition of Components of Wool and Hair

(Grams per 100 g. of protein)

Component	Epicuticle	Epicuticle	Scales	Cuticle (Hair)	Presumed cuticle	Cortical membranes, epicuticle	Medulla, resistant cortex (hair)	Spindle cells	Acid insoluble cortex (hair)	Paracortex, epicuticle	α-keratose	Spindle cells	Alkali-soluble derivatives	Orthocortex	Acid-soluble fraction
Reference	Schuringa (69)	Golden (27)	Geiger (22)	Lustig (45)	Ward (83)	Golden (27)	Lustig (45)	Lindley (42)	Lustig (45)	Golden (27)	Golden (27)	Ward (83)	Lindley (42)	Golden (27)	Ward (83)
Constituent															
Nitrogen	13		15.2	14.6			14.2	14.5	15.3			16.1	13.4		13.4
Sulfur	2.4		5.4	5.9			4.6	7.5	5.3			3.63	4.25		1.33
Amino acids															
Glycine	Increased relative to serine	0.7			4.0	3.2				2.7	6.7	3.6		9.1	6.9
Alanine		0.3			4.5	1.6		3.0		1.3	1.1	3.6	3.4	1.9	2.0
Valine		2.1			6	1.7		4.2		2.4	1.9		4.5	4.0	
Leucine		0.8			7.2	6.2		4.9		8.7	6.8	9.0	11.5	7.6	5.4
Isoleucine					3.1							4.0			2.4
Phenylalanine		0.3		1.5	2.2	1.5	3.2	1.2	1.7	1.9	1.1	2.9	2.7	1.5	4.4
Proline		2.5			8.5	5.0		9.1		5.6	6.3	6.6	1.1	8.0	4.1
Serine	Less	5.8	11.2	7.4	9.8	5.4	4.5	17 appr.	3.5	10.5	10.3	7.5		10.0	9.3
Threonine		2.0		3.8		2.1	5.6		6.8	5.6	6.6			8.2	
Tyrosine	Present		3.3	1.4	3.2		2.1	2.4	2.0			4.2	3.5		5.4
Aspartic acid		24.7			4.8	3.9				5.0	8.1	7.2		12.0	4.8
Glutamic acid		27.7			13	11.1				14.0	16.5	16.5		18.6	6.4
Arginine	As in whole wool	3.2	4.8	4.0	7.9	11.2	7.6	7.0	8.3	15.6	10.8	10.1		6.6	6.6
Lysine	Increased	1.4			3.6							3.6			2.3
Histidine	Much increased			0.6			1.2		1.3						
Tryptophan				0.2			0.3		0.3						
Cystine	3.5	13.5	20.3	19	0	6.1	12	25.2	16	17.1	13.3	10.6		9.8	2.5
Methionine				1											
Lanthionine	4.4														

It is clear that the composition has some relation to the solubility properties. Most directly, the cross-linking disulfide bonds may be considered to prevent solution by effectively increasing the molecular weight to "infinite" values. The evidence in support of this inference is the number of solubilization procedures involving reagents shown or presumed to act by disulfide bond cleavage. However, even with disulfide bonds broken, keratins are not freely soluble in dilute aqueous solutions. In view of the high proportion of polar groups, notably hydroxyl, amide, guanidyl, and carboxyl, it appears anomalous that this should be so. The explanation that is usually suggested is that these polar groups are hydrogen-bonded with one another in such a way as to stabilize the keratin structure and to be relatively inert or inaccessible to dilute aqueous reagents. Nevertheless, dilute aqueous reagents swell and plasticize keratins to a considerable extent. The exact detail of conditions restricting solution of reduced keratins is evidently an interesting unsolved problem. In spite of various experimental evidence that the more compact β structure, which is stabilized also by hydrogen bonding between peptide groups of adjacent polypeptide chains, is less soluble than the α form, feather, with crystalline portions in the β form, is consistently the most readily solubilized keratin (36).

An additional feature that imposes important restrictions on keratin solubilization is the existence of resistant, insoluble films around the fiber as a whole and around microscopic structural elements (50). The composition of these membranes is without much doubt different from that of the average wool protein. Schuringa and his co-workers characterize the membranous epicuticle as cross-linked protein (79) possibly with a certain amount of lanthionine and a cystine content that may be lower than average for wool (69).

b. Resistance to Hydrolysis. The resistance of keratins to hydrolysis in the presence of acids, bases, and enzymes is controlled to a considerable extent by limitations of swelling and solubility. The more obvious factors have been demonstrated experimentally. Thus enzyme action is favored (relatively slightly) by extreme mechanical subdivision (73), more by severance of disulfide bonds (41), by supercontraction, which disorganizes the more regular parts of the fiber structure, and by hydrotropic agents, such as urea and phenol, that tend to swell the fiber and lessen interaction between neighboring polypeptide chains (40).

A protein that is relatively resistant to hydrolysis might be supposed to have a relatively small proportion of peptide bonds specifically susceptible to given hydrolyzing conditions. (Sanger has included a summary of hydrolytic specificities in a recent review (66)). On the other hand, where resistance to hydrolysis is evaluated—as in textile fibers—by maintenance of strength, it might be supposed that absence of even a few unusually sus-

ceptible links would be imperative. As a matter of fact keratins, if wool may be taken as a typical example, are not strikingly resistant to acid hydrolysis (66). Both edestin and casein have longer half-lives, measured by the liberation of α-amino groups, than wool in 10 N hydrochloric acid at 37°C. Lysozyme is comparable. The abundance already noted of serine and threonine residues in keratins definitely makes *individual* peptide chains specially liable to acid hydrolysis. Experimental evidence also shows that bonds between glycine, aspartic acid, or glutamic acid residues and those of neighboring amino acids are hydrolyzed especially rapidly. These residues, too, are abundant in wool. Thus, about two of every five amino acid residues in wool would be expected to form peptide bonds unusually susceptible to acid hydrolysis. This chemical evidence makes the maintenance of reasonable wool fiber strength through usual processing operations of carbonizing and dyeing, in which the fiber is exposed to hot acid, rather surprising and of great interest, both practically and theoretically.

Although cystine, serine, threonine, and arginine are destroyed to some extent in alkaline hydrolysis, peptide bonds involving serine and threonine are more stable in alkali than in acid. Simple peptides of mono-amino, mono-carboxylic acids are hydrolyzed at similar rates in acid and alkali. Acid peptides are somewhat stabilized in alkali by their negative charge. In spite of these apparently favorable chemical factors wool is seriously degraded by alkali under milder conditions of concentration, temperature, and time than by acid.

Finally, enzyme specificities with respect to chemical structure of individual polypeptide chains cannot be expected to limit the attack of pepsin or trypsin on keratin proteins. The action of papain (5) is to liberate amino groups of several of the more abundant residues, notably serine, threonine, glycine, aspartic acid, and glutamic acid, among others. Of the better characterized proteolytic enzymes, chymotrypsin is probably the most restricted by chemical specificity in its attack on wool. Even so, it should be able to split about one in every ten peptide bonds. Accordingly it appears that the attack of hydrolytic agents generally—acids, bases, and enzymes—on keratins is not limited by chemical factors known to control stability of individual protein chains. The relative stability of the natural keratin fibers toward acid and their easy degradation by alkali suggest that in these highly cross-linked structures weak links in the peptide chains are less critically important than the cross-links, both primary (disulfide) and secondary (hydrogen bond). The polar amino acids that labilize individual polypeptide chains may be supposed to stabilize the structure as a whole through participating in hydrogen bonding between chains. Access of reagent to a particular bond is restricted by this mechanism. Restriction of movement about the bond also reduces its reactivity.

It is therefore apparent that individual keratin polypeptide chains are not particularly chemically resistant or inert. A better nomination for a standard of protein inertness would be elastin. This material has high proportions of proline and valine, which form peptide bonds resistant to hydrolysis, few residues of hydroxylic amino acids to introduce weak points, few acid and basic groups as reactive centers, and a low mean residue weight favoring structural compactness. Lacking these natural advantages, keratin structures achieve a working degree of mechanical and chemical stability by an extensive system of cross-bonding.

The preceding paragraphs call attention to the seeming paradox of the relative stability of keratins considering their high content of functional groups that have confirmed and well marked labilizing effects. Furthermore, the composition suggests a structure more stable in alkali than in acid, in contrast to experience. Kirkwood (36a) has recently estimated considerable attractive forces between polypeptide chains as a result of the fluctuations in number and configuration of mobile protons in the system. Maximum attraction would result from conditions permitting the greatest number of possible available resting sites for the greatest possible number of ionizable protons. This interesting mechanism provides a means of rationalizing some of these puzzles of the relation of properties to composition. For instance it provides an additional positive function for the various polar groups in stabilizing keratin structure by their provision of additional sources and resting sites for mobile protons. This additional mechanism differs from the relatively well known hydrogen bonding previously considered in that it has a long range action and does not demand a fixed, critical relation among the binding proton and specific bound groups. This mechanism accounts for the swelling, solubilizing action of alkali by the elimination of movable bonding protons. Relative resistance to acids is an indication of continued availability of ample mobile protons and excess reversible bonding sites at quite low pH's. Perhaps the most interesting application and test of the importance of this mechanism in keratin structure will be the prediction of the results of chemical modification of wool on the basis of changes in the supply of protons and available binding sites.

13. *Summary: Analytical Characterization of Keratins*

Keratins are natural fibrous materials consisting almost entirely of proteins stabilized by relatively frequent primary and secondary valence cross-links between neighboring polypeptide chains. The known primary valence cross-links are the disulfide bonds. All keratins and most keratin components are accordingly richer in cystine than are most other proteins. The strongest secondary valence cross-links consist of hydrogen bonds between the polar side groups of residues of amino acids, in particular

serine, threonine, glutamic acid, aspartic acid (or the corresponding amides of these acids), and arginine. Keratins have characteristically high contents of serine and arginine. (Many proteins besides keratins have high contents of glutamic acid.)

The mean amino acid residue weight of keratins is much higher than those of silk fibroin and the collagens. It is comparable to those of the other fibrous proteins myosin and fibrin(ogen), and in the lower end of the range characteristic of corpuscular proteins.

Finally, keratins have lower contents of proline plus hydroxyproline residues than the collagen family of fibrous proteins. Keratins have higher contents of proline than most corpuscular proteins, silk fibroin, and the more nearly related fibrous proteins myosin and fibrin(ogen).

The mean residue weight and proportion of amino acid residues, proline and hydroxyproline, affect the compactness and arrangement of the protein chains in the solid state in a way that is rationally related to chain spacings and configurations proposed to account for the evidence from X-ray and infrared analyses.

References

1. Alexander, P., Zahn, H., and Haselmann, H. (1951). *Textile Research J.* **21**, 236.
2. Auber, L. (1950–51). *Trans. Roy. Soc. Edinburgh* **62**, Part I, 191.
3. Barritt, J., and King, A. T. (1933). *J. Textile Inst.* **24**, T119.
4. Bekker, J. G., and King, A. T. (1931). *Biochem. J.* **25**, 1077.
5. Blackburn, S. (1950). *Biochem. J.* **47**, 443.
6. Block, R. J. (1938). *Cold Spring Harbor Symposia Quant. Biol.* **6**, 79.
7. Block, R. J. (1951). *Ann. N. Y. Acad. Sci.* **53**, 608.
8. Block, R. J. (1951). *J. Soc. Cosmetic Chem.* **2**, 235.
9. Block, R. J., and Bolling, D. (1951). "The Amino Acid Composition of Proteins and Foods," 2nd ed., Charles C. Thomas, Springfield, Ill.
10. Block, R. J., and Vickery, H. B. (1931). *J. Biol. Chem.* **93**, 113.
11. Bonsma, F. N. (1931). *J. Textile Inst.* **22**, T305.
12. Consden, R. (1949). *J. Textile Inst.* **40**, P814.
13. Consden, R., and Gordon, A. H. (1950). *Biochem. J.* **46**, 8.
14. Consden, R., Gordon, A. H., and Martin, A. J. P. (1949). *Biochem. J.* **44**, 548.
15. Cuthbertson, W. R., and Phillips, H. (1945). *Biochem. J.* **39**, 7.
16. Das, D. B., and Speakman, J. B. (1950). *J. Soc. Dyers Colourists* **66**, 583.
17. Eckstein, H. C. (1935). *Proc. Soc. Exptl. Biol. Med.* **32**, 1573.
18. Ellis, W. J., Gillespie, J. M., and Lindley, H. (1950). *Nature* **165**, 545.
19. Fraser, R. D. B. (1953). *Biochim. et Biophys. Acta* **12**, 482.
20. Fraser, R. D. B., and Rogers, G. E. (1953). *Biochim. et Biophys. Acta* **12**, 484.
21. Friend, J. A., and O'Donnell, I. J. (1953). *Australian J. Biol. Sci.* **6**, 630.
22. Geiger, W. G. (1944). *J. Research Natl. Bureau Standards* **32**, 127.
23. Gillespie, J. M., and Lennox, F. G. (1953). *Biochim. et Biophys. Acta* **12**, 481.
24. Giroud, A., and Leblond, C. P. (1951). *Ann. N. Y. Acad. Sci.* **53**, 613.
25. Goddard, D. R., and Michaelis, L. (1934). *J. Biol. Chem.* **106**, 605.
26. Goddard, D. R., and Michaelis, L. (1935–36). *J. Biol. Chem.* **112**, 361.

27. Golden, R. L. (1954). Thesis. Princeton University.
28. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
29. Graham, C. E., Waitkoff, H. K., and Hier, S. W. (1949). *J. Biol. Chem.* **177**, 529.
30. Gralén, N., Lagermalm, G., and Philip, B. (1951). *Textile Research J.* **21**, 234.
31. Happey, F., MacRae, T. P., and Weston, G. J. (1953). *Nature* **172**, 673.
32. Haselmann, H., and Zahn, H. (1951). *Melliand Textilber.* **32**, 1.
33. Horio, M., and Kondo, T. (1953). *Textile Research J.* **23**, 373.
34. Inskip, L. W. (1951). *J. Am. Chem. Soc.* **73**, 5463.
35. Jones, C. B., and Mecham, D. K. (1943). *Arch. Biochem.* **2**, 209.
36. Jones, C. B., and Mecham, D. K. (1943). *Arch. Biochem.* **3**, 193.
36a. Kirkwood, J. G. (1954). *in* "A Symposium on the Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.), Johns Hopkins Press. Baltimore, Md.
37. Koltun, W. L., Waugh, D. F., and Bear, R. S. (1954). *J. Am. Chem. Soc.* **76**, 413.
38. Lang, J. M., and Lucas, C. C. (1952). *Biochem. J.* **52**, 84.
39. Leblond, C. P. (1951). *Ann. N. Y. Acad. Sci.* **53**, 464.
40. Lennox, F. G. (1952). *Australian J. Sci. Research* B **5**, 189.
41. Lennox, F. G., and Forss, H. M. (1953). *Australian J. Biol. Sci.* **6**, 118.
42. Lindley, H. (1947). *Nature* **160**, 190.
43. Lindley, H. (1948). *Biochem. J.* **42**, 481.
44. Lustig, B., and Kondritzer, A. A. (1945). *Arch. Biochem.* **8**, 51.
45. Lustig, B., Kondritzer, A. A., and Moore, D. H. (1945). *Arch. Biochem.* **8**, 56.
46. Mahadevan, V. (1950). *Arch. sci. physiol.* **4**, 379.
47. Martin, A. J. P. (1946). *in* "Fibrous Proteins," p. 1, Society of Dyers and Colourists, Bradford, England.
48. Mercer, E. H. (1949). *J. Textile Inst.* **40**, T640.
49. Mercer, E. H. (1952). *Sci. Monthly* **75**, 270.
50. Mercer, E. H. (1953). *Textile Research J.* **23**, 388.
51. Mercer, E. H., and Olofsson, B. (1951). *J. Polymer Sci.* **6**, 261.
52. Mercer, E. H., and Olofsson, B. (1951). *J. Polymer Sci.* **6**, 671.
53. Middlebrook, W. R. (1949). *Nature* **164**, 501.
54. Middlebrook, W. R. (1949). *Nature* **164**, 321.
55. Middlebrook, W. R. (1951). *Biochem. et Biophys. Acta* **7**, 547.
56. Middlebrook, W. R. (1952). Personal communication.
57. Olcott, H. S. (1943). *Proc. Soc. Exptl. Biol. Med.* **54**, 219.
58. Olcott, H. S., and Fraenkel-Conrat, H. (1947). *J. Biol. Chem.* **171**, 583.
59. Olofsson, B. (1951). *Medd. Svenska Textilforskningsinst., Göteborg.* No. **14**, 57 [*Chem. Abstr.* **46**, 2807f (1952)].
60. Olofsson, B., and Gralén, N. (1947). *Proc. 11th Intern. Congr. Pure Appl. Chem., London* **V**, 151 (publ. 1953).
61. Pasynskiĭ, A. G., and Chernyak, R. S. (1950). *Doklady Akad. Nauk S.S.S.R.* **73**, 771 [*Chem. Abstr.* **45**, 685a (1951)].
62. Ripa, O. (1953). *Textile Research J.* **23**, 776.
63. Ripa, O., and Speakman, J. B. (1950). *Nature* **166**, 570.
64. Rudall, K. M. (1952). *Advances in Protein Chem.* **7**, 253.
65. Sanger, F. (1949). *Biochem. J.* **44**, 126.
66. Sanger, F. (1952). *Advances in Protein Chem.* **7**, 1.

67. Schuringa, G. J., Algera, L., Isings, J., and Ultée, A. J., Jr. (1952). *Experientia* **8**, 57.
68. Schuringa, G. J., Isings, J., and Ultée, A. J., Jr. (1952). *Biochem. et Biophys. Acta* **9**, 457.
69. Schuringa, G. J., Schooneveldt, C. J., and Ultée, A. J., Jr. (1952). *Biochim. et Biophys. Acta* **9**, 506.
70. Sidey, D. J. (1931). *J. Textile Inst.* **22**, T370.
70a. Simmonds, D. H. (1954). *Australian J. Biol. Sci.* **7**, 98.
71. Speakman, J. B. (1949). *in* "Fibre Science" (J. M. Preston, ed.), Chapter 16, The Textile Institute, Manchester, England.
72. Speakman, J. B. (1952). *Melliand Textilber.* **33**, 823.
73. Stahl, W. H., McQue, B., and Siu, R. G. H. (1949). *J. Biol. Chem.* **177**, 69.
74. Stary, Z. (1924). *Z. physiol. Chem.* **136**, 160.
75. Textile Research Institute (1953). Wool Research Project Final Report of Work on RMA Contract No. A-1s-30034, Princeton, N. J.
76. Tibbs, J. (1952). Cited by Speakman, J. B., *Melliand Textilber.* **33**, 823.
77. Toennies, G., and Homiller, R. P. (1942). *J. Am. Chem. Soc.* **64**, 3054.
78. Tristram, G. R. (1953). *in* "The Proteins" (H. Neurath and K. Bailey, eds.) Vol. I, Part A, Chapter 3, Academic Press, New York.
79. Ultée, A. J., Jr., Schooneveldt, C. J., and Schuringa, G. J. (1953). *Biochim. et Biophys. Acta* **10**, 590.
80. Ward, W. H. (1952). *Textile Research J.* **22**, 405.
81. Ward, W. H. (1948). *Abstr. Papers, 114th Meeting Am. Chem. Soc., Washington, St. Louis, and Portland*, p. 4F.
82. Ward, W. H., and Binkley, C. H. (1949). *U. S. Patent* 2,474,339.
83. Ward, W. H., Binkley, C. H., and Snell, N. S., Manuscript in preparation.
84. Ward, W. H., High, L. M., and Lundgren, H. P. (1946). *J. Polymer Research* **1**, 22.
85. Woodin, A. M. (1954). *Biochem. J.* **57**, 99.
86. Woods, E. F. (1952). *Australian J. Sci. Research* **5A**, 555.
87. Wool Industries Research Association (1954). Publication No. 199, Report of the Director of Research for 1953–54, Leeds, England.
88. Wilkerson, V. A., and Tulane, V. J. (1939). *J. Biol. Chem.* **129**, 477.